Oral effects of radiotherapy in head and neck cancer patients aetiology and pathophysiology linked to management

Gonzalez Agurto, Maria

Awarding institution:
King's College London

ORAL EFFECTS OF RADIOTHERAPY IN HEAD AND NECK CANCER PATIENTS; AETIOLOGY AND PATHOPHYSIOLOGY LINKED TO MANAGEMENT

Thesis submitted for degree of
Doctor of Philosophy

by

Maria Gonzalez Agurto

King's College London

December 2019

Abstract

Radiotherapy (RT), the mainstay treatment for head & neck cancer (HNC), is associated with salivary gland dysfunction. Intensity-modulated radiotherapy (IMRT) helps mitigate the severity of side effects. This is of particular note with oral mucositis being a very frequent side effect of head and neck cancer radiation therapy treatment having a clinical impact on patients being a limiting factor for cancer treatment, compromising its effectiveness. In addition, there is a lack of adequate treatment to reverse, control or prevent this side effect

The aim of this study was to analyse and evaluate the longitudinal nature of IMRT oral side effects and changes in nine salivary protein composition, as well as their possible associations, in order to develop a clinical and laboratory-based model, built on these connections, for the prediction of the incidence and severity of oral mucositis.

Samples were obtained from 40 head and neck cancer patients prior to IMRT (T0) at Guy's Hospital London in the Special Dental Care Unit, afterwards they were seen at six months post IMRT (T1) and 12 months post IMRT (T2). Unstimulated whole saliva samples were used to quantify flow rate and were analysed for total protein content using the Bicinchoninic Acid Assay (BCA), Periodic Acid Schiff (PAS) staining determined glycoprotein concentration, including mucin 5B and mucin 7, α-amylase activity was determined by kinetic assay, targeted Enzyme-Linked Immunosorbent Assays (ELISA) were used to quantified IgA, albumin cystatin S, acidic proline-rich peptides and statherin. Coomassie Brilliant blue was employed to detect carbonic anhydrase VI protein (CA VI) band.

In agreement with previous studies using conventional RT, IMRT resulted in decreased saliva flow rate and composition, along with xerostomia, taste variation reported by patients and oral mucositis score showed a significant, transient alteration.

Significant reductions in total protein secretion rate were identified for individuals on IMRT compared to baseline. Protein concentration for mucin 5B and mucin 7 were significantly elevated, concentration levels of IgA did not vary at any time point following IMRT. α-amylase activity and cystatin S were statistically decreased post-IMRT. Albumin concentration was significantly increased at T1, PRP and statherin reduction was not significant post IMRT.

There were reported significant associations among protein composition, secretion rate and this reduced flow rate as well as dry mouth feeling reported by patients and taste acuity. Additionally, it was observed significant association between oral mucositis onset /severity and saliva total protein concentration and secretion rate along with specific salivary proteins mucin 5B, mucin 7 and IgA as a part of the mucosal protection barrier and hydration layer, as well as amylase that play a role in bacterial colonization, cystatin S as an antibacterial protein and part of pellicle, and albumin as marker of inflammatory processes. These associations between clinical and biochemical data allowed the determination of potential cases that can serve as a reference for monitoring oral response to therapy. The current study proposed the use of novel salivary protein predictive model for the early diagnosis and potential severity of oral mucositis in head and neck cancer patients based on the analysis of ROC curves. The specific proteins concentration and secretion rate cut off points were selected regarding their sensitivity and specificity to target this condition and classify patients susceptible to develop severe mucositis prior to IMRT in a routine clinical situation.

Acknowledgements

First and foremost, my heartfelt gratitude goes to my first supervisor Professor Avijit Banerjee, for guiding me throughout my doctorate. My sincere thanks also go to my second supervisor, Professor Timothy Watson, for his help and support throughout my time at King's College.

Dr Ken Bruce and for guiding me through the early days of research as well as Dr Garrit Koller for his guidance, support and inspiration throughout the project.

My sincere gratitude to my postgraduate coordinator, Dr Karen Liu, for her support, and encouragement.

I would also like to thank all the members of the Salivary Research Lab. In particular Professor Guy Carpenter for his unceasing support, encouragement and friendship. You have all made my time at King's College enriching, inspiring and truly memorable, as well as enabling me to make lifelong friends.

Dr Mary Burke and everyone in the Special Dental Care Department for their help and support.

Professor Michael Fenlon for his advice and experience, specially from the clinical perspective was invaluable.

Mrs Fiona Warburton, for her valuable statistical advice and assistance.

UANDES university for their support which made possible to finish this endeavour.

Last but not least, I would like to thank my parents, my dad, my mum, and sister, for their support, patience, love and their belief in me. Specially, to Pedro you might be the next one (If you choose this endeavour).

Finally, a special thanks to my second family in London the Bozorgis, without their understanding and support, this PhD would have been impossible to complete.

List of Presentations

Oral Presentations

The 96th General Session of the IADR and Pan European Regional Congress on July 25-28, 2018 in London, England.

British Society for Oral and Dental Research (BSODR) Annual Scientific Meeting 2019, from 3rd-5th September, in Leeds, England.

Table of Content

Table of Figures

Table of Table

List of Abbreviations

BCA Bicinchoninic Acid Protein Assay

CiTAS Taste changes induced taste alteration scale

CTCAE Common Terminology Criteria for Adverse Events

CSLPS-IMRT Contralateral Superficial Lobe Parotid-sparing IMRT

DTT Dithiothreitol

EORTC QLQ European Organisation for Research and Treatment of Cancer Quality of Life questionnaires

Gy Gray

HN Head and Neck

HNC Head and Neck Cancer

HNSCC Head and Neck Squamous Cell Carcinomas

HADS Hospital Anxiety and Depression Scale

HRQOL Health-Related Quality of Life

IgA – Secretory Immunoglobulin A

IMRT Intensity Modulated Radiotherapy

LDS Lithium dodecyl sulphate

LENT-SOMA Late Effects of Normal Tissue Subjective Objective Management Analytical

MPL microplicae

MUC 5B mucin 5B

MUC7 mucin 7

OM Oral Mucosa

PAS Periodic acid Schiff's

PARSPORT phase III multi-centre randomised controlled trial of parotid sparing IMRT in patients with head and neck cancer.

PG Parotid Gland

PLUNC palate lung and nasal epithelium clone protein

PRX patient-reported xerostomia

QOL Quality of Life

RT Radiotherapy

RTOG Radiation Therapy Oncology Group

RTOG index Radiation Therapy Oncology Group grading system

RTOG/EORTC Radiation Therapy Oncology Group/European Organization for the Research and Treatment of Cancer

SCC squamous cell cancer

SDS PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SELDI-TOF-MS surface-enhanced laser desorption/ionization time of flight mass spectrometry

SG Salivary gland

SLG Sublingual gland

SMG Submandibular gland

SMS Stimulated whole mouth saliva

SR secretion rate

SS – Sjögrens syndrome

3D-CRT three-dimensional conventional radiotherapy

T0 Time point 0

T1 Time point 1

T2 Time point 2

TP – Total Protein

TP SR – Total Protein secretion rate

UWMS Unstimulated whole mouth saliva

WHO World Health Organization

XQ Xerostomia

Chapter 1

1.1 Introduction

In the treatment of advanced head and neck cancer, radiotherapy (RT) remains a mainstay, either as a monotherapy to control tumour size prior to surgery, or in conjunction with chemotherapy. Despite the high efficacy of localised radiotherapy in the treatment of tumours, adjacent tissues and structures can suffer significant injury resulting in off-target effects, that may be severe, debilitating and treatment-limiting. The salivary glands are among the normal tissues often affected in patients with head and neck cancers (HNC) as the RT beam path, in most instances, must traverse these sensitive tissues with high radiation weighting (absorption and susceptibility of tissues). One of the most significant long-term complication of radiotherapy is a progressive loss of salivary gland function, leading to quantitative and qualitative losses in saliva, recognised clinically as radiation-induced xerostomia (dry mouth)(Vissink et al., 2010; Baskar et al., 2012; Duarte et al., 2014; Almståhl et al., 2019).

In addition to injury to the major glands, oral complications may be related to permanent cell damage induced by the toxic effects of radiation. DNA of cells in the tissues surrounding the tumour site is affected, impairing cell division and resulting in cell death or deterioration of functional characteristics. For instance, cumulative doses >30Gy will produce degeneration of salivary gland acinar cells along with inflammation and fibrosis. Even though cells have slow turnover rates (circa 60 to 120 days) the changes in volume and composition start immediately after RT. Further RT is known to produce modification of connective tissues causing acute apoptosis of fibroblasts and vascular tissues preceding the damage in the epithelial cells resulting in induced mucosal injury and reduction of healing particularly in mucosal tissue (Sonis, 2004a; Rieger et al., 2012; Turner et al., 2013).

All of these complications are interconnected; salivary gland hypofunction induces a significant reduction in salivary flow rate, causing xerostomia, which will hamper speech, swallowing and altering taste perception as well as producing nocturnal oral discomfort (Wijers et al., 2002; Vissink et al., 2003; Nutting et al., 2011; Vissink et al., 2015). A reduced salivary flow rate is usually accompanied by an altered saliva composition, leading to an

increased risk of oral diseases and hastening progression of dental caries. It is well established that along with healthy diet and oral hygiene, saliva is important in maintaining tooth integrity through mineral and bacterial homeostasis acquired enamel pellicle and antibacterial activity and host protection (Hannig et al., 2017; Pitts et al., 2017).

Intensity modulated radiation therapy (IMRT) was developed to mitigate the toxic effects of RT on the tissues surrounding the tumour site, minimising salivary gland hypofunction. Studies found lower instances of xerostomia reported by patients as well as improvements in salivary flow rate and quality of life parameters related to xerostomia in head and neck cancer patients (Nutting et al., 2011). However, around 40 % of head and neck cancer patients still experience moderate to severe sensations of xerostomia along with a reduced salivary flow rate following IMRT (Vissink et al., 2010; Belstrøm et al., 2016; Janus et al., 2017).

In addition to these chronic oral manifestations, one of the most acute side effects of IMRT is oral mucositis, which may be life threatening and requires intensive treatment. Studies established that approximately 80% of patients undergoing RT will develop oral mucositis, characterized by the presence of extensive, deep and painful ulcers affecting any oral mucosal surface. This intense inflammatory response may severely impair or prevent eating, swallowing, drinking and speaking. Clinical management is targeted towards the managements of symptoms, such as opiate-based analgesics and parenteral feeding through nasogastric tubes. No effective treatment to prevent or eliminate oral mucositis has been found to date. Severe cases of oral mucositis will need hospitalisation which can delay cancer treatment and lead to dose / fractioning alteration, thus limiting the effectiveness of the cancer therapy (Logan et al., 2007; Sonis, 2011; Epstein et al., 2012; Duarte et al., 2014; Villa and Sonis, 2015; Franco et al., 2017; Maria et al., 2017; Jung et al., 2019). Much of the literature assessing RT side effects have been observational, documenting only the oral status of a small number of head and neck cancer patients after RT from a quality of life perspective. Even though salivary flow rate was measured in some studies it was used only to determine hyposalivation and xerostomia(Nutting et al., 2011; Memtsa et al., 2017; Almståhl et al., 2019). Only a small number of investigations evaluated pH and buffer capacity in saliva after RT, albeit without a benchmark (Möller et al., 2004; Sim et al., 2018) and to date there has been no saliva biochemical components analysis or link to clinical oral complications

It is not possible to draw any definitive conclusions on salivary biochemical composition changes post-RT due to the limited number of longitudinal studies (short follow-up period) which have been mostly retrospective, observational and cross sectional with a smaller number of patients recruited. Further each study has used a different saliva collection technique and collection time along with a lower quantity of biochemical components analysed (Funegård et al., 1994; Almståhl et al., 2001; Eliasson et al., 2005; Hannig et al., 2006; Vidotto et al., 2010; Dijkema et al., 2012; Laheij et al., 2015).

Thus, the long-term nature of the changes in salivary protein composition and correlations between individual salivary proteins and their corresponding clinical xerostomia, taste alteration, carious lesion, and oral mucositis outcomes remain unexplored.

1.2 Overview

Overcoming limitations of previous studies, the following research project will assess the most significant acute and long-term complications of RT that affect HNC patients: oral mucositis, dry mouth feeling, taste alteration and carious lesion incidence, all of which negatively affect cancer treatment outcomes and the quality of life of cancer survivors. This project was developed with an aim to study any possible correlations / links between the oral sequelae of RT and the negative clinical outcomes listed, in order to help the development of clinical management protocols for HNC patients to mitigate the effect on care and patient comfort and its delivery in healthcare systems.

The project comprises of a longitudinal cohort study of HNC patients correlating biodata of biochemical salivary parameters, clinical outcomes and oral health status over a period up to 12 months post-RT, focusing on baseline pre-IMRT (T0), 6 months post-IMRT (T1) and up to 12 months post–IMRT treatments (T2). The ultimate goal is to identify possible predictive markers in the saliva at T0 to assist with evaluation of oral mucositis severity in individual HNC patients prior to start of treatment so that treatment can be adapted for such high-risk patients individually, to either prevent or minimise the severity of this consequence of RT.

The study was performed in accordance with the Declaration of Helsinki. Patients were recruited at Guy's Dental Hospital, London, UK in the Special Dental Care Unit from the pool

of diagnosed HNC patients prior to undergoing IMRT, having gained full written consent. Forty patients were seen pre-IMRT (T0). From this cohort, 38 were seen at six months post IMRT (T1) and 33 at 12 months post IMRT (T2).

The first results chapter includes the oral and dental assessments performed on all participant including DMFT(S) indices, dry mouth feeling taste change reported from a simplified questionnaire and oral mucositis severity. Severity of any mucositis present was assessed using the WHO oral mucositis scale. Unstimulated whole mouth saliva was collected at each time point by passive drooling for 10 minutes. Flow rate was measured by weighing the sample tube before and after collection. Saliva samples were labelled and frozen at -80º C on the 17th floor laboratory at Guy's Dental Hospital.

The discussion of the results in this chapter looks at whether salivary flow rate was statistically significantly affected following IMRT, any correlation with reported of xerostomia and taste changes, any significant reduction in number of teeth present in the mouth and variations in the quantity of carious lesions at T1 and T2, compared to T0. The results obtained from the clinical assessment showed clear links between UWMS flow rate, dental assessments of oral health, dry mouth sensation and taste perception, to salivary gland dysfunction. On the other hand, oral mucositis presence was related to a reduced UWMS flow rate at T1 and T2 as well as to a dry mouth feeling reported by patients at both time points.

The second results chapter provides insight into the RT effects on salivary proteins chosen due to their previously established roles in mucosal protection from desiccation, provision of lubrication and microbial protection as well as maintaining enamel integrity. The saliva samples were used to quantify total protein concentration and secretion rate, in order to assess the amount of protein entering the oral cavity. Nine specific salivary proteins were investigated, all of which are directly associated with oral health, mucosal and teeth protection, antibacterial, rheological properties, remineralisation and microbial homeostasis. They can act as possible markers of the two most common acute and late RT side effects - oral mucositis and radiotherapy caries, as well as other oral symptoms such as dry mouth and taste perception. Total protein concentration was quantified by BCA assay. Periodic Acid Schiff staining determined glycoprotein concentration, including mucin 5B (MUC5B) and mucin 7 (MUC7). Coomassie brilliant blue determined Carbonic Anhydrase VI pixel intensity,

ELISA assays quantified IgA, cystatin S, statherin, acidic proline-rich peptides and albumin. α-amylase activity was determined by kinetic assays.

Significant alteration was observed in composition and secretion rate, starting with total protein, as well as mucins, amylase, albumin, cystatin S and carbonic anhydrase following IMRT T1 and T2 in comparison with baseline results recorded at T0. This chapter analyses any association between radiation-induced changes in salivary composition with the clinical dental assessment, taste alteration and xerostomia reported by patients. The total number of teeth present, and number of surfaces affected visibly by caries were significantly affected, in association with changes in total protein concentration and secretion rate, mucin 5B and 7, amylase and cystatin S at T1 and T2. Dry mouth feeling was significantly associated to a reduced total protein secretion rate, and to an increased mucin 5B and 7 concentration in saliva, these changes alter saliva viscosity reducing its lubrication capacity impeding to reach oral mucosa and be retained on it, this will make mucins uncapable of forming a gel and covering oral epithelium to protect it from dehydration, resulting in to an altered mouth feel. Taste alteration reported by patients was only significantly associated to CAVI concentration and secretion rate.

The clinical relevance of changes in salivary quantity and quality are those that salivary properties directly influence lubrication of the oral cavity, hydration of the oral mucosa, alter normal mouth feel and contribute to overall homeostasis.

The final chapter studies the possible relationship between salivary proteins content and specifically those responsible for mucosal pellicle protection, lubrication, hydration and the onset and severity of oral mucositis. In order to achieve this, aim a longitudinal regression panel was used. Subsequently, statistical analytical ROC curve model was applied. This statistical model evaluates the sensitivity and specificity of the marker proteins to define their capacity to predict the risk and severity of oral mucositis in head and neck cancer patients undergoing IMRT, prior starting the treatment.

These analyses revealed that specific salivary proteins amylase unit and secretion rate assessed at T0 may be a possible predictive marker for mucositis development during radiotherapy.

Regarding severity of oral mucositis mucin 5B concentration/secretion rate as well as total protein concentration and secretion rate analysed at T0 may be a possible determining factor of high severity of oral mucositis occurrence during the cancer treatment.

It is important to remark that there is no report in the literature studying the longitudinal correlation between IMRT induced changes in salivary proteins with oral mucositis development and severity. Therefore, this study is first of its kind that has attempted to find these correlations in forty head and neck cancer patients across twelve months post IMRT.

These findings highlight the potential importance of a patient cantered approach as a clinical endpoint focusing on improving preventive management regimes for HNC at risk of developing severe oral mucositis. Identifying HNC patients at high risk of severe mucositis and consequently informing the oncology and health care team could possible play a major role in improving their quality of life.

Oral mucositis represents a significant clinical challenge and causes major burden for HNC patients as mentioned earlier.

The relevance of these finding is regarding patient morbidity and suffering, both of which have not substantially improved in the past decades, patients continue receiving only palliative care due to a lack of an effective treatment to reverse oral mucositis. Thus, the prevention, early management and control of oral mucositis is vital, so that the incidence and degree of this condition can be reduced (Sonis, 2004a, 2011; Epstein et al., 2012; Duarte et al., 2014; GB et al., 2015; Franco et al., 2017; McCullough, 2017; Almståhl et al., 2019).

On the other hand, the impact in the cost of care may be substantial due to repetitive hospitalization for pain management, parenteral feeding and secondary infection treatment. Ultimately resulting in unplanned cancer treatment interruption, delays or cancellations linked to recurrence of disease and a subsequent lowering of survival rates (Sonis, 2011; Villa and Sonis, 2015; McCullough, 2017; Münstedt et al., 2019).

By having a possible indicator of the severity of oral mucositis, might have imperative impact on patient care, involving fewer hospital visits, decreasing number of emergency room visits, reducing the health care practitioners additional time managing of mucositis-related issues consequently reducing significant costs for the NHS.

1.3 Aim and Objectives

Saliva quantity and quality performs a substantial role in oral homeostasis, constantly covering oral mucosa and teeth to ensure lubrication, hydration and microorganism colonization. Therefore, altered protein composition as well as a reduced flow rate are associated with frequency and severity of various oral and mucosal diseases, fungal infections and dental caries.

It has been reported in literature, the effects of radiotherapy in the oral cavity regarding to patient's quality of life, however not many researchers have focused on salivary gland function regarding salivary protein composition and secretion rate. Therefore, long term effects of IMRT on saliva composition and potential associations with oral side effects remains unclear.

The aim of this project is to perform a well-controlled and integrated longitudinal study of HNC patients correlating biodata of biochemical parameters, clinical parameters and oral health status, to overcoming the limitations of previous studies, that examined the different side effects of radiotherapy of HNC patients (reduced number, retrospective, observational, short term follow up, lack of detailed clinical assessment, focused into treatment of side effects).

The first objective of this study will be to perform quantitative analysis of oral/ dental status of HNC patients pre and post IMRT and analyse the possible associations between the clinical outcomes.

Followed by assessing salivary gland function of HNC patients pre and post IMRT by analysing protein concentration and secretion rate and investigate the possible associations between these proteins and clinical outcomes.

Next objective is to determine the possible association between selected proteins concentration and secretion rate and oral mucositis outcomes after IMRT, and therefore, the final objective is to evaluate whether protein concentration or secretion rate of saliva is a reliable predictor of mucositis presence and severity.

1.4 Null Hypothesis

There will be no correlation between salivary biochemical composition and oral side effects of radiotherapy in the HNC patients before and after radiotherapy.

1.5 Literature Review

1.5.1 Saliva

1.5.1.1 Physiological Role of Saliva and Composition

Saliva can be said to affect all functions of the mouth; the complex biofluid that makes up saliva is an important component of several processes including homeostasis, maintenance of the symbiotic balance in the oral cavity, as well as other vital functions of oral physiology. Saliva has been defined as a complex matrix containing proteins, enzymes and electrolytes, including an important sink of ions maintaining tooth regeneration and repair (Hunter, 2013; Hannig et al., 2017; Pedersen et al., 2018)

A key characteristic of saliva is its ability to envelop oral surfaces as a non-Newtonian fluid, without being easily washed off. Teeth and mucosal surfaces are immediately covered by salivary proteins, forming a functional barrier via the adsorption of these components (Kilian et al., 2016; Pedersen et al., 2018).

Saliva is composed mainly of water (99.5%) and inorganic substances (0.2%), such as electrolytes, whereas proteins represent only 0.3% of salivary composition. However, the composition may vary depending on flow rate, circadian rhythms, systemic diseases and other factors such as during eating and drinking (Vidotto et al., 2010; Proctor, 2016).

Total protein concentration represents approximately 1 - 2 milligrams per ml of saliva, the main constituents of this group are glycoproteins, enzymes, immunoglobulins and peptides,

which are responsible for multiple protective functions(Denny et al., 2008; Schuurhuis et al., 2016).

Alterations in salivary secretion rates are associated strongly with changes in oral homeostasis, as a result of ageing, systemic diseases, medication and medical treatments (Pedersen et al., 2018; Proctor, 2016).

1.5.1.2 Saliva Flow Rate

The average daily volume of saliva secreted is between 500 - 600 ml (Proctor, 2016). The major salivary glands (parotid, submandibular and sublingual) secrete 90% of the total saliva, with the remaining 10% being secreted by minor salivary glands (Tschoppe et al., 2010).

Unstimulated Whole Mouth Saliva (UWMS) is a mixed secretion that comes from every salivary gland. The contribution of each salivary gland without stimulation is approximately 60% via submandibular glands, 25% from parotid glands, with the sublingual and minor salivary glands combining to contribute 7 – 8% of the total salivary composition (Hunter, 2013; Pedersen et al., 2018). UWMS flow rate is an indicator of salivary gland function, being 0.2 - 0.3 ml/min. (NAVAZESH, 1993; Tschoppe et al., 2010; Chaudhury et al., 2015; Pedersen et al., 2018).

A reduction that goes below these baseline levels is defined as “hyposalivation” – a functional loss where the salivary flow is reduced, ultimately leading to an altered chemical composition of the saliva (Tschoppe et al., 2010; Chaudhury et al., 2015)(Tschope et al., 2010; Chaudhury et al., 2015).

1.5.1.3 Salivary Glands

Salivary glands are exocrine glands developed from epithelial cells, major salivary glands proliferating from the epithelial buds developing into mesenchymal tissue. This process is guided by molecular signals during embryonic development (Proctor 2016).

Salivary glands are composed of two types of epithelial cells: acinar and ductal cells. Most salivary proteins are secreted by acinar cells, whereas ductal cells only represent a minor contribution regarding the total protein secretion rate (Carpenter, 2013a; Proctor, 2016; de Paula et al., 2017).

Within acini, different cell types determine which of the two types of salivary proteins are synthesised by each gland, mucin or non-mucin content. Serous cell acini secrete a watery fluid that is rich in salivary enzymes, via exocytosis(Humphrey and Williamson, 2001). Mucous cells secrete viscous glycoproteins and mucins that form mucus, which serous cells lack (de Paula et al., 2017).

Parotid glands are purely serous in nature, while submandibular and sublingual glands contain both mucous and serous cells. This cellular conformation helps determine the main type of saliva secreted by each gland (Denny et al., 2008). Parotid glands secrete saliva which contains mainly water, enzymes (α-amylase) and a small concentration, if any, of mucin glycoproteins (these glycoproteins are responsible for its viscoelastic properties). This parotid saliva is capable of moistening both hydrophobic and hydrophilic surfaces, which is important for mastication, moistening and swallowing of different types of food with diverse properties.

Submandibular glands are seromucous glands, comprising 10% mucous cells and 90% serous cells (Hunter, 2013; Schipper et al., 2007). Finally, sublingual and minor salivary glands are formed mainly by mucin producing acinar cells, secreting approximately 80% of total mucins present in saliva, a high viscosity fluid that prevents mucosal dehydration. At the same time this saliva presents a high elasticity and low shear rate which permits retention and adhesion to oral mucosa. Additionally, minor salivary glands and parotid produce secretory immunoglobulin A (IgA). The minor mucous salivary glands are located in the submucosa, distributed throughout the oral cavity excluding the hard palate and gingivae. Their main function is mucosal protection and lubrication given their specific protein production. They also have an important role in preventing bacterial infections in teeth such as caries lesion. The secretory products of the salivary glands, after being synthesized intracellularly, are secreted into the ductal system via intercalated ducts, striated ducts, interlobular and main excretory ducts in each salivary gland. This complex network is capable of modifying hypotonic saliva into isotonic fluid before of conducting this into the oral cavity (de Paula et al., 2017; Carpenter, 2013a).

1.5.1.4 Salivary Gland Innervation

Salivary secretions, in humans, are controlled by the parasympathetic and sympathetic nerves that reach acinar cells, ductal cells, myoepithelial cells and blood vessels. Sympathetic nerves reach mainly parotid and submandibular glands, whilst sublingual and minor salivary glands are reached by adrenergic innervation. Animal studies have revealed that different neurological stimulation can vary salivary composition and volume via the nervous system. The parasympathetic system is responsible of electrolyte secretion and normal salivary flow rate, protein secretion is preferentially activated by sympathetic stimulation, however there is a substantial cross-talk and synergism between the two regulatory pathways. Parasympathetic stimulation and peptidergic stimulation can regulate the levels of production of mucin-rich saliva from sublingual and minor salivary glands(de Paula et al., 2017; Proctor, 2016; Mandl and Ekberg, 2019; Castro et al., 2013). Sympathetic innervation is related to the amount of protein secreted under reflex taste stimulation which is responsible for the dry mouth feeling(Proctor and Carpenter, 2007). Finally, adrenergic stimuli from the sympathetic system can cause changes to protein secretion levels in parotid and submandibular glands (Mandl and Ekberg, 2019; Castro et al., 2013; Hunter, 2013; Schipper et al., 2007; Proctor, 2016).

There are different mechanisms for certain proteins and specific immunoglobulins, such as IgA, which is secreted by plasma cells present inside salivary glands. IgA is carried across the cells into the lumen of the acinus and an epithelial polymeric immunoglobulin receptor is added to this molecule on the basolateral membrane of acinar and ductal cells. This receptor works as a transporter, dividing this complex when gets saliva (Proctor, 2016; Brandtzaeg, 2009). Certain proteins are released by vesicular secretion, without any external stimulus, which may result in accumulation of these molecules in the ductal system. Other proteins are synthetized and deposited in granules, only released by stimulation(Proctor, 2016).

1.5.1.5 Salivary Proteins

Salivary protein composition and flow rate are crucial in oral health, being involved in a wide range of functions in order to maintain homeostasis. Proteins can be related to rheological properties of saliva, antimicrobial activity as well as probiotic activity, all of which form a system that defends the oral cavity (Schipper et al., 2007; Pinna et al., 2015; Pedersen et al., 2018).

Moreover, salivary flow rate and biochemical composition are important in the clearance of nutrients and bacteria (Dawes et al., 2015). Any changes to the flow rate affect the concentrations of mucin content, glycoproteins or Proline-Rich Proteins (PRP), which have been directly associated with carious lesion development, diminishing the protective effect of tooth mineralization homeostasis (Zussman et al., 2007; Peros et al., 2011;Hemadi et al., 2017; Cardoso et al., 2017).

Salivary mucins are high molecular weight glycoproteins that are essential in determining saliva's viscoelastic properties, which in turn, regulates the lubrication and hydration of oral mucosa creating a membrane (Proctor, 2016) mucin 5B (MUC 5B) (2-4 x 104 kDa) and mucin 7 (MUC 7) (130-180kDa) both comprise almost 25% of the total protein concentration. Both mucins are secreted mainly by minor and sublingual glands (>70%), with submandibular glands contributing only 20% of total production (Chaudhury et al., 2015; Frenkel and Ribbeck, 2015). Structurally, mucin 5B and mucin 7 are negatively charged, highly glycosylated proteins. Their protein backbone contains serine and threonine amino acid sequences, capable of binding to sialylated or sulphated oligosaccharides (O-glycosilated tandem repeat attached to the protein core). These carbohydrate chains give mucins the capacity of being hydrophilic polymers capable of binding to water, preventing desiccation, providing anti-proteolytic properties and specific attachment sites for pathogens (Castro et al., 2013; Hannig et al., 2017).

Furthermore, mucin 5B is the primary gel-forming mucin in the oral cavity (Frenkel and Ribbeck, 2015) due to its high amino acid content along with multiple glycan chains. Specifically, mucin 5B can bind calcium as well as interacting with proline-rich proteins, statherins, and histatins. In contrast, mucin 7 lacks gel-forming properties (Xu et al., 2016) and exists essentially as monomers or dimers. Mucin 7 is capable of attaching secretory IgA

at sites close to the mucosal pellicle, increasing its concentration close to the oral epithelium. Additionally, it is capable of binding antibacterial proteins in order to increase protein concentration levels in different locations throughout the oral cavity, as well as playing an important role in preserving the viscoelastic properties of saliva together with the ability to contribute to bacterial clearance (Dawes et al., 2015; Vijay et al., 2015; Frenkel and Ribbeck, 2015; Hemadi et al., 2017).

These protective barriers play an important role in preventing demineralisation, stimulating remineralisation, maintaining a neutral pH, protecting opposite surfaces from abrasive forces during mastication, speaking and swallowing (Castro et al., 2013; Gibbins et al., 2014; Dawes et al., 2015 ;Hemadi et al., 2017; Mandl and Ekberg, 2019). On soft tissue surfaces, the barriers form a level of protection against dehydration, whilst keeping tissues lubricated and protecting underlying tissues from proteolytic attack (Gabryel-Porowska et al., 2014; Ligtenberg and Almståhl, 2015; Hemadi et al., 2017).

MUC7 can also play a part in maintaining oral cavity homeostasis by binding with antibacterial elements, such as acidic and basic PRPs, statherins and histatin 1. It is believed that mucins may act as carriers for these antibacterial molecules transporting them to different locations within the oral cavity, increasing their concentration in mucosal or acquired enamel pellicle or forming a protective system around these proteins in order to inhibit the proteolytic degradation of these molecules (Frenkel and Ribbeck, 2015).

Certain studies have shown the potential capability of mucins to aggregate bacteria in order to increase their chance of removal during swallowing, as well as determining bacterial colonization on oral surfaces, by attaching certain microbial species on specific glycans, such as sialic acid and blood-group antigens, even though these studies do not distinguish between MUC5B and MUC7 (Levine et al., 1978; Frenkel and Ribbeck, 2015). Additionally, it is specifically known that MUC5B is capable of binding specific bacteria in a biofilm formation in order to reduce their virulence (Frenkel and Ribbeck, 2015; Hemadi et al., 2017). Others have studied the spectrum of antimicrobial and antifungal activity of MUC7, due to a non-glycosylated region at the N-terminus of the apomucin molecule. The presence of O-glycans forms a large protease-resistant region providing binding spots for commensal bacteria and pathogens (Xu et al., 2016), even finding it capable of interacting directly with them to facilitate their removal from the oral cavity (Frenkel and Ribbeck, 2015; Hemadi et al., 2017).

It also has potent killing capabilities against a variety of fungi and both gram-positive and gram-negative bacteria, including Escherichia coli, Staphylococcus aureus, Candida albicans and Cryptococcus neoformans(Frenkel and Ribbeck, 2015; Hemadi et al., 2017). MUC5B is only capable of modulating the virulence of Candida albicans pathogens without killing them and this could potentially be the reason why candida can be present in the oral cavity without causing candidiasis (Frenkel and Ribbeck, 2015).

MUC5B is capable of creating a highly concentrated network across oral surfaces in order to keep the mouth lubricated, suggesting a possible relation between dry mouth feeling and mucin 5B concentration (Lee et al., 2007). Further studies have correlated this mucin concentration with xerostomia, concluding that mucin 5B is an effective lubricant in the mouth, rather than only correlating this symptom with the amount of whole mouth saliva present in the mouth (Dijkema et al., 2012).

Both mucins are among the main components of mucosal pellicle, along with secretory IgA, as their presence is found in a higher concentration than in saliva(Hannah L. Gibbins et al., 2014; Dawes et al., 2015; Hemadi et al., 2017). Mucins are related to mucosal tissue protection against injuries along with Epidermal growth factor (EGF) (Pedersen et al., 2018). Moreover, MUC7 and MUC5B, along with other proteins such as statherin, histatin, acidic proline-rich proteins, α-amylase and lysozyme are part of the acquired enamel pellicle (Hannig et al., 2017). These findings all suggest that mucins are a vital component for preventing carious lesion development, whether it be via oral defence binding, reducing bacterial virulence, or maintaining opportunistic pathogens in the oral environment without causing diseases (Frenkel and Ribbeck, 2015).

Albumin, produced in the liver and carried into the mouth as an ultra-filtrated serum, represents more than 50% of all plasma proteins (Meurman et al., 2002). Albumin is well-established as an indicator of plasma leakage in the oral cavity, typically as a result of inflammatory processes; therefore it can act as a marker for oral mucositis in relation to radiation therapy in head and neck cancer patients (Schenkels et al., 1995; Almståhl et al., 2001; Meurman et al., 2002; Chiappin et al., 2007; Messana et al., 2008). Additionally, it is capable of binding to hydroxyapatite forming part of enamel acquired pellicle and coats soft oral surfaces playing an important role in lubrication (Schenkels et al., 1995; Chiappin et al., 2007; Cone, 2009; Messana et al., 2008; Hannig et al., 2017). α-A-amylase, secreted by parotid

glands, is one of the most abundant enzymes in the mouth and considered to be a marker of parotid gland function (Almståhl et al., 2001). Its primary function is starch digestion by catalysing the hydrolysis of glycogen starch- related polysaccharides(Granger et al., 2007). Similarly, to MUC5B, it is also capable of binding oral bacteria, such as Streptococcus *gordoni,* S. *mitis* and S. *oralis* (Dawes, 2008). α-amylase binds to the membrane of bacteria in order to aid their clearance from the oral cavity, therefore decreasing the risk of dental caries (Borghi et al., 2017; Picco et al., 2017).

α-Amylase is capable of selectively binding to bacterial colonies in the oral cavity (Nikitkova et al., 2013; Gao et al., 2016; Borghi et al., 2017; Cardoso et al., 2017), providing additional glucose for the formation of tooth biofilm that maintains the normal flora (Gao et al., 2016; Borghi et al., 2017; Cardoso et al., 2017). This biofilm production and caries formation is linked to this binding of α-amylase to bacteria and teeth, as α-amylase enables the necessary starch hydrolysis for microorganisms, metabolism and lactic acid.

Carbonic Anhydrase VI (CAVI) is an enzyme secreted by parotid and submandibular glands that plays a fundamental role in controlling taste sensation and taste bud growth, as well as development and/or maintenance, especially fungiform papillae. CAVI facilitates the interaction between food particles and taste buds (Denny et al., 2008; Hunter, 2013). In order to counteract the excessive acidity of the tooth surface biofilm, CAVI is activated and catalyses the most important buffer reaction in the oral environment; the carbonic dioxide equilibrium in saliva (Öztürk et al., 2008; Frasseto et al., 2012; Zwier et al., 2013; Cardoso et al., 2017; Pedersen et al., 2018), in order to increase bicarbonate salivary levels especially with acidic foods. Moreover, this enzyme plays a role in reducing tooth demineralization by activating this buffer mechanism that neutralizes the acidic pH of biofilm (Kimoto et al., 2006; Cardoso et al., 2017).

Statherin is a phosphoprotein secreted from parotid, submandibular and sublingual glands, which plays a multifunctional role in the oral cavity, maintaining calcium homeostasis, preventing its precipitation and crystal growth, as well as keeping saliva supersaturation in order to stimulate the remineralisation process. It is an acquired enamel pellicle early precursor, also playing an important role in promoting the initial microbial colonization of tooth surfaces and present antifungal properties (Niemi and Johansson, 2004; Li et al., 2004; Proctor et al., 2005; Gorr, 2009; Vergeer et al., 2009; Izumi et al., 2015; Vukosavljevic et al.,

2014; Dawes et al., 2015). In addition, Statherin has lubricating properties, preventing teeth from chipping and wearing down during mastication (Schipper et al., 2007; Carpenter, 2013a).

Acidic PRPs secreted by parotid and submandibular glands contain between 25-42% of proline amino-acids (Carpenter, 2013a).

Acidic PRP, statherin and other phosphorus-containing proteins are capable of binding calcium, thereby maintaining a higher concentration than teeth (supersaturated saliva), in order to prevent dissolution of teeth and to avoid its precipitation, especially at the cervical margins of teeth causing calculus (Carpenter, 2013a; Hemadi et al., 2017). Acid and basic PRPs are part of the acquired enamel pellicle due to their calcium hydroxide-binding properties. These proteins participate in oral bacterial clearance, binding bacteria, fungi and viruses towards the stomach. Furthermore, these proteins have a polyphenol binding affinity, acting as polyphenol precipitators (tannins included) in the perception of astringency (Fábián et al., 2015).

Cystatins are a family of seven low molecular weight proteins secreted primarily by submandibular glands. Cystatins can act as a form of microorganism control, whether it be control of lysosomal cathepsins, inhibition of bacterial cysteine proteases, binding bacterial lipopolysaccharides, adenovirus or herpes simplex 1 infectivity and viral replication (Messana et al., 2008; Fábián et al., 2012; Magister and Kos, 2013; Aroonsang et al., 2014; Fábián et al., 2015; Hemadi et al., 2017; Martini et al., 2017). Cystatins also are a part of the acquired enamel pellicle formation and play an important role in tooth remineralisation processes (Gao et al., 2016; Martini et al., 2017; Hemadi et al., 2017). More specifically, cystatin S inhibits the action of endogenous, bacterial and parasitic protozoan proteases, binds bacterial lipopolysaccharides and seems to exert direct immunomodulatory effects(Martini et al., 2017; Fábián et al., 2012).

Secretory IgA is the main antibody of saliva and is secreted by plasma cells located inside the glands under conditions of stress, disease or inflammation. Healthy oral cavities would typically find minimal to no diffusion of IgA into saliva. On the oral epithelium it is highly concentrated forming part of the mucosal pellicle (Carpenter, 2013a; H. L. Gibbins et al., 2014; Hannah L. Gibbins et al., 2014). IgA plays an antibacterial role by interfering with the adherence of microbial flora to tooth surfaces, inhibiting bacterial metabolism, neutralizing

bacterial toxins and enzymes as well as the agglutination of bacteria. These all aid in strengthening the anti-microbial action of saliva (Fábián et al., 2012; H. L. Gibbins et al., 2014; Gao et al., 2016; Hemadi et al., 2017).

Additionally, secretory IgA and α-amylase are resistant to bacterial proteolytic enzymes (Schipper et al., 2007) and some studies have identified IgA as a protective mechanism in children with early childhood caries (Hemadi et al., 2017). Immunoglobulin A (IgA) concentration increases in elderly people in UWMS as well as this non stimulated whole saliva secretion rates were reduced with age (Eliasson et al., 2006).

1.5.1.6 Salivary Interaction with Oral Tissues

Saliva protective functions are related directly to its capacity of interact with soft and hard tissues in the oral cavity by forming a complex layer that is capable of moisten and lubricate these surfaces along with being capable to remain on the surfaces (Hannig et al., 2017).

Residual saliva (RS) is a term used to refer to a salivary film covering oral structures and preventing the oral mucosa from feeling dry. Its composition is dependent on a mixture of saliva from every gland coating all the surfaces in the mouth, with each location's thickness varying and with its wetting capacity a determinant of the dry mouth feeling in correlation with protein concentration levels (Pramanik et al., 2010; Chaudhury et al., 2015). Perception of dry mouth has been correlated to insufficient mucosal wetting and altered unstimulated whole saliva composition (Lee et al., 2007; Pramanik et al., 2010; Chaudhury et al., 2015). The mucosal pellicle is a thin layer (70-100 micrometres) covering soft tissues of the oral cavity in order to aid with lubrication and moisturizing. Mucosal pellicle attachment to the epithelial cell surface results in it not being able to be cleared with water or other ionic washes (H. L. Gibbins et al., 2014; Hannah L. Gibbins et al., 2014; Hannig et al., 2017). It is formed by a complex network layer of salivary proteins that are structurally different from enamel pellicle. It is capable of retaining high volumes of water due to its composition which is mainly a glycoprotein protein (MUC5B, MUC7 and secretory IgA) hydrogel (H. L. Gibbins et al., 2014; Dawes et al., 2015; Hannig et al., 2017; Hemadi et al., 2017). Further in-vitro studies have also found more proteins, such as cystatin S and PRPs included in its structure (Dawes, 2008; H. L.

Gibbins et al., 2014; Hannig et al., 2017). Still inconclusive, (Lee et al., 2007; H. L. Gibbins et al., 2014; Hannig et al., 2017) some studies have found that subjects with a dry mouth sensation experience a decreased thickness of this pellicle, (less than 10um) (Dawes, 2008).

This protective layer regulates the early adhesion and colonization of bacteria on the oral mucosa as well as regulating oral microbiota conformation (Pedersen et al., 2018). Saliva contact with hard tissues is mainly by the interaction between specific proteins and teeth surfaces in order to create a protective layer to ensure a high concentration of calcium, buffering capacity and lubrication. It is well established that the main function of the enamel pellicle is the protection of teeth from erosion and abrasion (Kielbassa et al., 2006; Hannig et al., 2017). The structure and thickness of acquired enamel pellicle changes depending on the location and time of formation, composed typically by a two-phase structure of an electron-dense basal layer and a globular overlying structure. These structures are formed by salivary proteins, the main components being MUC5B, statherin, acidic proline-rich proteins, histatins, α-amylase and lysozyme; that are therefore crucial for re-mineralisation/demineralisation of the enamel (Meurman et al., 2002; Hannah L. Gibbins et al., 2014). MUC5B, PRPs, histatin and statherin are the first proteins to bind to enamel due to the negative charges that come from the acidic side-chains. The main function of the pellicle is the regulation of the calcium phosphate concentration in the oral cavity, impeding crystal growth, keeping calcium supersaturated in saliva close to the tooth surface thereby controlling dental erosion. Acquired enamel pellicle promotes the adhesion of different bacteria to hydroxyapatite on tooth surfaces (Buzalaf et al., 2011; Magalhães et al., 2011; Vukosavljevic et al., 2014; Hannah L. Gibbins et al., 2014; Hannig et al., 2017; Hemadi et al., 2017; Mutahar et al., 2017). In the mature enamel pellicle, more protein-protein interactions are observed, where MUC7 and MUC5B are capable of binding α-amylase to this layer (Mutahar et al., 2017; Hannig et al., 2017).

It is well established that lubrication is one of the most important functions of saliva, reducing the friction between two surfaces during movement. A lack of this property will lead to irritation and ultimately, destruction of soft and hard tissues. This function is directly related to salivary films, enamel and mucosal pellicle and the presence of saliva (H. L. Gibbins et al., 2014; Hannig et al., 2017). Literature has suggested that lubrication properties of saliva depends on its viscosity as well as on the physical characteristics of the adsorbed surface. It

has been proposed that viscosity is more important in soft tissue lubrication than in hard surfaces. Glycoproteins and proteins are responsible of lubrication properties of saliva such as the mucins, especially 5B. Its viscoelastic and protective properties are vital in coating and wetness of oral surfaces. In addition, statherin, α-amylase, proline-rich glycoproteins and acidic proline-rich proteins are involved in this process (Hahn Berg et al., 2004; Proctor et al., 2005; Schipper et al., 2007).

1.5.2 Head and Neck Cancer (HNC)

Head and neck cancers are the sixth most common cancer in the world representing 4-5% of all malignancies (Joshi, 2010; Gualtero and Suarez Castillo, 2016). These cancer varieties include oral, nasal cavity, salivary glands, paranasal sinus, oropharynx and larynx cancers, however approximately 90% of oral and oropharyngeal cancer is squamous cell carcinoma, representing the most common cancer in the head and neck region (Chi et al., 2015). Primarily affected by risk factors such as drinking or smoking habits, human papillomavirus (HPV) is considered an aetiologic factor for oropharyngeal squamous cell carcinoma, more specifically HPV 16 (Chi et al., 2015).

Reduced quality of life is common among these cancer sufferers, not only with the direct side effects associated with radiation therapy (RT), but also their psychological, emotional and social burdens (Wissinger et al., 2014).

1.5.3 Radiotherapy

Radiotherapy (RT) is a physical and localized treatment to control tumours. As the first choice of treatment for the majority of head and neck cancer patients, RT is mainly delivered by an external beam in a fractioned dose in order to preserve the normal tissues exposed (Baskar et al., 2012; Duarte et al., 2014; Almståhl et al., 2019). More than 50% of patients receive treatment delivered in fractions of 2 Grays (Gy) daily for 5 days per week until the end of their therapy dosage (mean cumulative dose 55-77 Gy) (Chao et al., 2001; Springer et al., 2005;Sennhenn-Kirchner et al., 2009; De Siqueira Mellara et al., 2014; Gao et al., 2015). Ionising radiation creates its therapeutic effect by interacting with the water molecules within cells to create free radicals that cause chemical damage, altering a cell's structure, chemical composition and functions (Breneman, 1994). By affecting cells directly, they can alter the DNA, therefore diminishing the division rate of tumour cells, eventually leading to autophagy causing cell death (Baskar et al., 2012; Strojan et al., 2017). Radiation effectiveness depends on total dose fractionation and radio sensitivity of targeted cells.

RT has been proven to induce late side effects to the head and neck, including salivary gland hypofunction (Richards et al., 2017) and results in dry mouth (xerostomia), reduced salivary flow rate and altered saliva composition (Nutting et al., 2011).

1.5.4 Radiotherapy Side Effects

Conventional radiotherapy is the cause of a wide range of adverse oral tissue reactions, such as permanent damage to the major and minor salivary glands, modification of connective and vascular tissues, chronic inflammation and its resulting effects. Side effects of radiotherapy can be categorised into two types, depending on the time of appearance (acute and late), that result in several oral pathologies during and after radiotherapy. Acute effects occur from the very beginning of treatment, whereas late effects occur after treatment has concluded to produce long term effects (3 months or more) (Vissink et al., 2003; Meurman and Grönroos, 2010; Walker et al., 2011; Gonçalves et al., 2014; De Siqueira Mellara et al., 2014; Lieshout and Bots, 2014; Laheij et al., 2015). Acute effects include mucositis, salivary gland

hypofunction and taste alterations, whereas late adverse effects are permanent salivary gland damage, caries and periodontal pathology, amongst others.

Salivary gland hypofunction induces a reduced salivary flow rate causing xerostomia, which not only affects speech and swallowing, but can also hasten the progression of dental caries (Wijers et al., 2002; Nutting et al., 2011; Belstrøm et al., 2016; Janus et al., 2017). Chronic inflammation and its cytotoxic effects on epithelial tissue also leads to mucositis, presenting large, irregular and deep ulcers within the mouth (Rieger et al., 2012; Turner et al., 2013; Gao et al., 2015). Hyposalivation after RT is a common side effect due to irradiation of salivary glands, producing acini cell or blood vessel damage. However, it is not clear whether the damage is caused by direct cytotoxic effects of radiation on secretory and ductal cells or indirectly affecting the vascular flood in the salivary gland by chronic inflammatory infiltration (Klein et al., 2014). Radiation doses and the volume of irradiated tissue have been directly correlated with salivary gland hypofunction, whereby cumulative doses exceeding 30 Gy have even caused permanent tissue damage (Klein et al., 2014).

Moreover, xerostomia, disrupted salivary gland function and altered biochemical composition of the saliva can be observed separately, but are usually interrelated as a side effect of RT. Altogether, in the long run, they will contribute to the increase in caries risk in these groups of patients (Chaudhury et al., 2015; Laheij et al., 2015; Sroussi et al., 2017).

1.5.4.1 Hyposalivation

Xerostomia affects more than 80% of head and neck radiotherapy patients (Tschoppe et al., 2010; Pinna et al., 2015; Villa and Sonis, 2016). A diminished salivary output results in an altered salivary biochemical composition along with a change in salivary viscosity (Tschoppe et al., 2010; Vissink et al., 2010; Riley et al., 2017; Pedersen et al., 2018). Unstimulated whole mouth saliva flow rate lower than 40-50% of its normal value is indicative of xerostomia and probably more than one salivary gland is affected by hypofunction. However, xerostomia may be a result of salivary composition changes specially mucins that are responsible of lubrication functions(Pedersen et al., 2018).

Such changes in saliva affect a patient's ability to chew and swallow, deteriorating their quality of life due to their inability to distinguish different food preferences, types, quality of nutrients and finally, to differentiate safe and dangerous foods (Tschoppe et al., 2010;Rieger et al., 2012; Pinna et al., 2015). Even though the prevalence of changes in taste after cancer treatment has been described, the mechanism involved is not well understood and there is a lack of literature to support the subjective changes in taste before and after radiotherapy, affecting threshold and or producing an alteration of taste perception (Epstein et al., 2016; Spotten et al., 2016; Epstein et al., 2019).

Taste requires an appropriate saliva flow rate to be perceived by taste buds throughout the oral cavity, oropharynx and dorsal surface of the tongue in order to evaluate the content of food and distinguish it from toxic elements and proper nutrients, preventing ingestion of these substances, making the food consumption a satisfactory experience connected positively with the ability to enjoy the flavour and texture of food (Epstein et al., 2019).

Dysgeusia or alteration in taste perception and loss of taste (ageusia) has been associated with damage in the gustatory system, ageing, xerostomia and Sjögren's syndrome. Head and neck cancer patients suffer loss of taste acuity and a metallic or bitter taste during the radiotherapy, ranging from 75-100% and may be related to the onset of mucositis. Over 90% of the patients that received a dose of 60Gy developed a relative taste loss. Taste impairment is related to radiation dose fraction size, volume and technique, starting during the first week of radiotherapy and progressing during the treatment reaching the maximum point in the fourth week. This taste dysfunction has been studied using survey methods by patient-reported outcomes. Additionally, hyposalivation and saliva quality may increase the taste disturbance, negatively affecting the quality of life of these patients, reducing treatment compliance, altering food intake, food choice, desire to eat, distress and interfering with daily functions. After radiotherapy, taste dysfunction may continue indefinitely due to damage to receptors and/or hyposalivation which will result in a reduced food particles dissolution, decreasing the molecule number able to reach taste receptors (Ruo Redda and Allis, 2006; Bressan et al., 2016; Epstein et al., 2016, 2019; Jham et al., 2009).

Furthermore, it can make the mouth feel dissimilar, with oral mucosa becoming dry and sticky increasing the risk of mucosal ulceration and injuries (Chao et al., 2001; Vissink et al., 2010; Pinna et al., 2015; Strojan et al., 2017) and food being difficult to swallow, which in the long

run, creates a nutritional deficiency that affects general health. Additionally, the lack of lubrication properties and wettability of saliva will result in speech problems, creating difficulties in communication. The resulting lack of social interaction has a negative impact on the general health and well-being of these patients (Vissink et al., 2010; Pinna et al., 2015; Chaudhury et al., 2015). Subjects with hyposalivation caused by radiation therapy showed differences in oral microflora composition compared with age-gender control patients (Almståhl et al., 2001), increasing its susceptibility to bacterial and yeast infection (Chaudhury et al., 2015). Hyposalivation subjects presented a similar number of tooth surfaces covered with plaque during the dental examination. The difference in bacterial composition was associated with an altered salivary protein concentration (Almståhl et al., 2001) reduced salivary flow rate, increased sugar intake and poor oral hygiene raising the risk of an unbalanced oral microbiome mainly dominated by the microorganisms associated with oral diseases (Kilian et al., 2016).

In addition, reduced flow rate leads to a reduced food clearance altering bacteria composition that can impact taste acuity (Epstein et al., 2016).

All the above will result in serious consequences for oral homeostasis, reducing important protective saliva functions, increasing caries risk leading to a tooth destruction and oral mucositis.

1.5.4.2 Radiation Therapy Related to Hyposalivation In Head and Neck Cancer

It is well established that there are many different causes for hyposalivation including a variety of systemic diseases; chronic autoimmune conditions, such as Sjögren's syndrome, lupus erythematosus, rheumatoid arthritis, scleroderma, hormonal causes (diabetes mellitus), neurologic causes (Parkinson's disease), anorexia, alcohol abuse, infections and depression. Additionally, there are more than 400 types of medications capable of reducing the salivary flow rate (Tschoppe et al., 2010).

Patients will develop signs of atrophic mucosa, erythema, angular cheilitis and oral ulcers, conditions that are associated with pain and changes in taste perception that may affect oral functions, thus leading to nutritional impairment, weight loss, isolation and depression,

stopping the treatment all together (Tschoppe et al., 2010; Osailan et al., 2012; Sroussi et al., 2017).

Roesink et al., (2001), studied 108 head and neck cancer patients before and after they were treated with conventional radiotherapy in order to correlate dose/volume/parotid gland function. The stimulated parotid flow rate was measured before radiotherapy, then 6 weeks, 6 months, and 1 year after radiotherapy. After 6 weeks of radiotherapy the salivary flow rate was significantly reduced compared with the base line (0.12mL/min), after 1 year the mean flow rate reached 0.20 mL/min still remaining under the base line mean flow (0.34 mL/min). A reduced flow rate was associated with increasing fraction of the irradiated parotid gland. There was no information regarding the general health of the patients, nor an oral assessment and the salivary flow rate before the cancer treatment was not compared with a control group.

Möller et al., (2004), studied 39 patients treated with radiotherapy alone or with surgery (n=36), where unstimulated and stimulated salivary flow rate, pH and buffering capacity were measured. Smoking and alcohol habits were recorded as well.

Unstimulated whole mouth saliva for all patients decreased gradually during irradiation from 45% to 21%, compared with the mean baseline. Similarly, for stimulated whole mouth saliva, the rate decreased from 39% to 14% showing a faster and steeper decrease in flow rate compared with unstimulated saliva. Stimulated whole mouth saliva mean flow rates decreased statistically and significantly during the entire study compared with unstimulated. The pH dropped after 1 week of radiotherapy, then started to increase until the end of the treatment reaching 7.05 for WSS, lower than the baseline. WRS pH was 6.97, marginally higher than before the cancer treatment. The buffering capacity of saliva dropped significantly from the first week of irradiation, reaching the minimum value after 3 weeks of treatment, maintaining this decrease until the end of RT. After 12 months, the buffering capacity was still significantly lower than baseline. It was concluded that radiotherapy induces severe hyposalivation, alteration of salivary pH, and a significant loss in salivary buffering capacity. There is a lack of analysis regarding the salivary components of saliva, there was no control group to compare before RT, no oral assessment of this group, nor oral disease and dry mouth reports.

Dijkema et al., (2008), evaluated 221 head and neck cancer patients treated with IMRT and conventional RT in a retrospective study. Stimulated parotid flow rates were measured before as well as 6 weeks, 6 months and 1 year after radiotherapy in order to evaluate the relationship between the mean dose and the parotid gland and the probability of complications for CRT and IMRT. Thus, a complication was defined as "stimulated parotid flow ratio <25% of the pre-treatment flow rate". Parotid gland complication after 6 weeks and 6 months was 1.4 times higher when IMRT was used, compared with CRT. One year after the radiotherapy was completed, there was no difference between the two types of RT. This retrospective study associated radiotherapy effects to parotid gland hypofunction, only measuring the flow rate. There was no analysis of the quality of saliva after radiotherapy.

Dijkema et al., (2010)multicentre study analysed 222 H&N cancers treated with conventional and intensity-modulated RT. Stimulated parotid gland flow rates were measured before the treatment and 1 year after RT. A flow ratio <25% of pre-treatment was defined as a complication. The aim was to assess the normal tissue complication probability (NTCP) of the parotid gland 1 year after radiotherapy. It was found that the Mean doses of 25–30 Gy were associated with 17–26% NTCP. Like the previous study, there was no sialochemistry analysis in order to assess the side effects of radiotherapy in parotid gland function.

Nguyen et al., (2018) compared conventional RT and IMRT in 96 patients with head and neck cancer, assessing hyposalivation by comparing stimulated flow rate before and after treatment, then correlating this with time, total dose received and unilateral and bilateral conventional RT. Additionally, the amount of RT received by the parotid gland was evaluated using Quantitative Analysis of Normal Tissue Effects in the Clinic (QUANTEC criteria), in order to predict the severity of hyposalivation after cancer treatment and to predict the risk of oral diseases and reduce the number of teeth extractions before radiotherapy. Patients that received bilateral conventional RT presented with a significantly reduced salivary flow rate compared with unilateral and IMRT after radiotherapy when contrasted with baseline measurement. At 12 months, the three groups showed a trend of salivary flow recovery, being the bilateral radiotherapy group the smallest compared with their baseline flow rate.

IMRT groups were not divided into bilateral and unilateral. There was no oral assessment before and during the follow-up to correlate with the hyposalivation in order to measure the risk of oral diseases. Additionally, only stimulated saliva was collected.

Memtsa et al., (2017), studied salivary flow rate and xerostomia in 60 head and neck cancer patients treated with conventional radiotherapy to correlate this with quality of life. The European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire-Core 30 (QLQ-C30), the Quality of Life Questionnaire Head and Neck Module (QLQ-H&N35) and the Greek version of the XQ questionnaire were applied at 4 timepoints before and after radiotherapy. Unstimulated and stimulated whole mouth saliva were collected and pH was measured, xerostomia was graded using Radiation Therapy Oncology Group (RTOG). Quality of life decreased significantly at 6 months after radiotherapy, XQ questionnaire scores were significantly lower when correlated with salivary flow rate. Salivary flow rate was 20% lower than baseline at 12 months after RT. Xerostomia was correlated with quality of life scores, but not with flow rate measures at 12 months. There was no analysis of protein composition of saliva before and after radiotherapy related with xerostomia. The study of salivary biochemical composition related with mucosal hydration is important in patients that present signs and symptoms of xerostomia.

Sim et al., (2018) evaluated xerostomia scores, stimulated salivary flow rates, pH and buffering capacity before, during and after radiotherapy (IMRT) in 15 head and neck cancer patients. Xerostomia scores were determined using the Radiation Therapy Oncology Group/European Organization for the Research and Treatment of Cancer (RTOG/EORTC). Unstimulated and stimulated saliva were collected pre and post radiotherapy. Xerostomia scores were significantly higher 2 years after radiotherapy, stimulated and unstimulated salivary flow rate decreased significantly after radiotherapy, together with pH and buffer capacity. Even though the variation of flow rate and pH was analysed before and after radiotherapy, the number of patients included in this study was low and there was no report of salivary composition.

Similarly, Almståhl et al., (2019) analysed the quality of life of 29 head and neck cancer patients pre and up to 24 months post-radiotherapy related to stimulated saliva secretion. Patients completed the European Organisation for Research and Treatment of Cancer Quality of Life questionnaires (EORTC QLQ-C30 and H&N35) and the Hospital Anxiety and Depression scale (HADS) at four time points: pre-treatment and at 6, 12- and 24-months post-radiotherapy. Stimulated saliva was collected for 3 min at every time point. Patients that showed signs of hyposalivation showed significant differences in their quality of life at every

time point after radiotherapy, presenting signs of insomnia, difficulties in swallowing, reduced social eating, reporting dry mouth and sticky saliva. Hyposalivation groups experienced poor everyday functioning and reported more symptoms related to salivary flow rate at 24 months, especially regarding eating and drinking impairments which resulted in social isolation. Additionally, this group had a reduced sense of taste at 24 months, describing that food is tasteless or different.

Salivary flow rate and composition are both related to oral health, due to all the protective functions of saliva in order to maintain integrity of soft and hard tissues.

As a side effect of radiotherapy, hyposalivation and an altered saliva composition are going to impair the antimicrobial, remineralising and buffering capacity of saliva, as well as the lubrication and hydration properties of saliva increasing the risk of developing oral diseases.

Most of the studies performed presented with an absence of information about participants, regarding systemic diseases, medication, oral diseases, caries risk, carious lesion numbers, localization, teeth/surface and dental treatment received. Only the salivary flow rate was assessed to determine salivary hypofunction as a result of radiotherapy and correlate with quality of life of head and neck cancer patients. None of the studies mentioned above performed any analysis of the total protein component or specific proteins regarding salivary microbial and host protection and viscoelastic / lubrication properties which could affect the capability of saliva to humectify the oral mucosa.

1.5.4.3 Radiotherapy Related to Taste Disturbance

Taste requires an appropriate saliva flow rate to be perceived by taste buds throughout the oral cavity, oropharynx and dorsal surface of the tongue in order to evaluate the content of food and distinguish it from toxic elements and proper nutrients, preventing ingestion of these substances, making the food consumption a satisfactory experience connected positively with the ability to enjoy the flavour and texture of food (Epstein et al., 2019).

A reduced salivary flow rate may affect the intensity of taste acuity as a result of a reduced capacity for dissolving food molecules, which reduces the number of these particles reaching taste receptors. Additionally, oral diseases such as carious lesions and candidiasis may

influence gustatory changes, especially in head and neck cancer patients who present xerostomia (Epstein and Barasch, 2010; Irune et al., 2014; Epstein et al., 2019).

Taste dysfunction is related to the development of mucositis and dysphagia during cancer treatment. These are acute side effects of radiotherapy which are dose-limiting, affecting care plan compliance, extension and results in increasing mortality and morbidity. In addition, these complications will affect nutritional status, appetite and food consumption, altering daily routine and leading to a reduced quality of life (Ruo Redda and Allis, 2006; Irune et al., 2014; Epstein et al., 2016, 2019). It is important to identify the side effects of radiotherapy in order to help cancer survivors to comprehend and manage these sequelae and thereby maximize the functions post-treatment and improve their quality of life.

Spotten et al., (2016), focused on assessing the prevalence and severity of distorted taste and smell perception and sensitivity after cancer treatment using a 'Taste and Smell Survey' (Heald et al.,1998) and nutritional status using versions of the scored Patient-Generated Subjective Global Assessment (abPG-SGA). Fourty non-head and neck cancer patients were assessed, 19 reported at least one sense affected after radiotherapy, along with dry mouth. Many patients reported anxiety about poor food variety and some of them reported reduced social activity due to these side effects which were significantly associated with quality of life in this group.

Similar results were obtained by (Zabernigg et al., 2010) taste alterations (TAs) and the association with quality of life in 197 non-head and neck cancer patients. Taste alteration was found in many patients and was associated positively to various aspects of quality of life such as fatigue and appetite loss. Additionally, a loss of eating pleasure was reported, which was associated with weight loss and diminished social interaction.

Both studies were performed in non-head and neck cancer patients, without any information related to oral status, hygiene habits, medication, systemic diseases, salivary flow rate and protein composition before and after radiotherapy.

Jin et al., (2018) studied the relationship between weight loss and taste alteration in 114 head and neck cancer (HNC) patients who were treated with intensity modulated radiotherapy. Using a scale to measure subjective taste alteration, weight and body mass index (BMI) were assessed at baseline, mid-treatment, post-treatment and two months after radiotherapy. The

prevalence of subjective taste alteration after radiotherapy was more than 75%, along with discomfort during food intake and weight loss as well as mean BMI. Although there was no correlation between weight lost and taste alteration. Similarly, to the previous studies, salivary flow rate and protein composition were not measured together with the oral and dental status of this group of patients after the radiotherapy. In addition, there was no report of dry mouth feeling.

Epstein et al., (2019) evaluated only five subjects with hematologic malignancy and nine head and neck cancer patients, who reported taste changes after treatment from 1-6 months. In order to test taste, a chemical gustometry for the primary taste qualities of sweet, sour, salty and bitter was used and umami flavour was assessed by using a modified Henke test. Patients' answers were selected from the following choices: sweet, salty, sour, bitter, spicy, and tasty, or no taste. Dry mouth was assessed using the NCI Common Terminology Criteria for adverse Events (CTCAE) 4.0 and the Scale of Subjective Total Taste Acuity (STTA) was used to describe the loss of taste function (from 0 to 4). Stimulated and unstimulated salivary flow rate was measured, defining hyposalivation in 0.1ml/min for unstimulated and 0.5-0.7 ml/min for stimulated. Head and neck cancer patients' taste function was reported as follows: grade 2 taste disturbance was 44%, grade 3 was 44%, and one patient reported grade 1. Regarding the gustometry test, four patients (44%) described no taste, three (33%) described an abnormal taste and two (22%) described moderate to severe reduction in sweet taste. Dry mouth was reported by all the patients in this group, showing grade 2 in 66% and grade 1 in 33%. A reduced salivary flow rate was observed in 67% of the patients. Some patients reported improvement in taste after treatment, but all had dry mouth. Despite the low number of subjects included in this study of taste acuity after radiotherapy, salivary flow rate was measured, and dry mouth was reported by patients in order to associate these factors. There is no information of oral status, smoking and drinking habits, oral diseases such as carious lesions, mucositis and candida during and after radiotherapy. In addition, salivary protein composition before and after radiotherapy was not analysed.

Martini et al., (2019)examined 31 head and neck cancer patients undergoing radiotherapy only or with chemotherapy. Taste changes were assessed using the (CT)-induced taste alteration scale (CiTAS), a scale based on 18 items to assess qualitative, quantitative and diet-related toxicities before and after radiotherapy (1w, 1month and 6 months), pain was

evaluated using a visual analogue scale from 0 to 10. A statistically significant difference ($p < 0.05$) was observed for reduced taste perception from baseline and at every time point, similarly for discomfort, metallic taste and presence of an unpleasant taste and for general taste alterations score.

Ogama et al., (2010) studied 117 head and neck cancer patients receiving radiation therapy. The relationship between radiation treatment at different dosages and adverse effects like dysgeusia, xerostomia, and oral mucositis resulting in appetite changes were analysed. Fluctuations in saliva production, analgesic use, frequency of oral care, subjects' characteristics and appetite were studied during the cancer treatment at 20, at 30, and 50 Gy. Xerostomia was assessed by observing the amount of saliva and patients reported saliva production. Mucositis was evaluated using 5-point Common Terminology Criteria for Adverse Events (CTCAE), v3.0. Appetite survey was applied and analgesic intake. At 50 Gy, it was seen that xerostomia, sensitivity of taste and oral mucositis influenced appetite. Xerostomia is related to a reduced flow rate and oral care. Mucositis is affected by analgesic use. At 50 Gy all patients presented dysgeusia. This shows the importance of controlling xerostomia, dysgeusia and oral mucositis through the proactive use of analgesics for pain in order to prevent appetite reduction, to increase patients' comfort, radiation therapy tolerance, QOL, and cancer treatment success. It is important to maintain oral care at this radiotherapy dosage.

Flavour is a complex interaction between many sensorial factors including a normal salivary flow rate, taste and it is connected to emotions evoking a sense of well-being. After radiotherapy, hyposalivation may contribute to taste impairment due to a lack of dissolution of the food particles. Additionally, altered saliva viscosity impedes the correct transport of the molecules to the taste receptors (Epstein and Barasch, 2010; Irune et al., 2014; Epstein et al., 2019). Taste is related to energy intake, water consumption, nutrients and protection from toxins and poison. Macro and micronutrients are vital in the maintenance of health and tissue repair, especially after oncology treatments. The capacity to appreciate food affects the quality of life of head and neck cancer patients (Epstein et al., 2016).

Taste alteration mainly influences the quality of life of head and neck cancer survivors regarding food selection, content evaluation, desire and oral intake of food, which leads to weight loss and nutritional implications. Changes in taste which have been evaluated by

patients are related mainly to quality of life. It is important to assess the changes in taste perception after radiotherapy and associate this with salivary gland hypofunction, examining the quality and quantity of saliva in order to correlate these outcomes with the possible role of saliva in taste changes in head and neck cancer patients in a long term follow-up.

There are a lack of studies that include patient evaluation and analysis of the salivary production and protein content from unstimulated whole mouth saliva related to taste disturbances reported by the patients. There is a deficiency of information about oral examination of this group of patients, reporting carious lesions, dental treatment, mucositis, candida, smoking and drinking habits, oral hygiene in head and neck cancer patients undergoing radiation therapy. Finally, microbial analysis would add valuable information related to infections which alter the oral homeostasis and influence taste disfunctions.

1.5.4.4 Radiotherapy Related to Xerostomia

Diminished salivary flow rate has been one of the most common acute side effects leading to a dry mouth feeling or xerostomia. Xerostomia affects more than 80% of head and neck radiotherapy patients (Tschoppe et al., 2010; Pinna et al., 2015; Villa and Sonis, 2016). A diminished salivary output results in an altered salivary biochemical composition along with a change in salivary viscosity (Tschoppe et al., 2010; Vissink et al., 2010; Riley et al., 2017; Pedersen et al., 2018).

However, xerostomia may be a result of salivary composition changes especially mucins that are responsible for lubrication (Pedersen et al., 2018).

Xerostomia is a common side effect after radiotherapy in head and neck cancer patients, associated to hyposalivation and altered composition of saliva, which may lead to oral mucosal dehydration and cause a dry mouth feeling (Wiener et al., 2010; Nguyen et al., 2018). Altered flow rate and composition increase the risk of tooth demineralization and reduce remineralization due to the lack of calcium and phosphate increasing the caries risk and tooth extractions, post-extraction osteoradionecrosis, candidiasis and mucosal ulcerations (Jensen et al., 2010; Epstein et al., 2012). Additionally, xerostomia is associated to functional

impairments in chewing, swallowing, speaking and mucosal sensitivity (Murphy and Deng, 2015).

Chao et al., (2001), prospective clinical study before and after 6 months of radiotherapy in 41 H&N cancer patients, objective and subjective xerostomia was tested in patients with head-and-neck cancers treated with intensity-modulated radiation therapy (IMRT) (n 27) and three-dimensional radiation therapy (n=14). Stimulated and unstimulated parotid saliva was collected before radiotherapy and 6 months after. Quality of life (QOL) was assessed with five questions regarding the patients' oral discomfort and eating/speaking problems. A correlation was seen between parotid mean dose and reduction of stimulated saliva flow rate after 6 months of RT. Also, stimulated and unstimulated saliva flow showed a significative correlation with eating/speaking functions at 6 months. The use of RT that is capable of sparing parotid glands showed an objective and subjective improvement of both xerostomia and QoL scores in head-and-neck cancers patients. The patients assessed for salivary flow rate were not compared with a control group and there was no analysis of the protein content to relate with xerostomia.

Vergeer et al., (2009), compared patient-rated xerostomia in 241 head and neck squamous cell carcinomas (HNSCC) treated with intensity-modulated radiation therapy (IMRT) and three-dimensional conventional radiotherapy (3D-CRT) using Radiation Therapy Oncology Group (RTOG) acute and late xerostomia and health-related quality of life (HRQOL). IMRT group resulted in both observer-rated and patient-rated xerostomia that was significantly lower than the two-dimensional radiotherapy, improving quality of life. However, 40% of patients still complained of xerostomia. No saliva was collected and there was no assessment of the salivary flow rate before and after the cancer treatment, nor was there any biochemical analysis of saliva.

Hawkins et al., (2018) studied patient-reported xerostomia related to quality of life in head and neck cancer patients using patient-reported outcome measures (PROMs) including patient-reported xerostomia (PRX). Xerostomia (XQ) and head-and-neck quality of life (HNQOL) questionnaires were answered by 252 head and neck cancer patients treated with bilateral IMRT at 8 time points until 60 months after IMRT.

Before radiotherapy, there was no register of these parameters. A positive correlation was shown between QOL and patient reported xerostomia and the radiation dose received by each gland.

Kawamoto et al., (2018) studied retrospectively the incidence of late subjective xerostomia (severe xerostomia, grade >=2) in 71 oropharyngeal and hypopharyngeal cancer patients undergoing conformal radiotherapy (3D-CRT) and contralateral superficial lobe parotid-sparing IMRT (CSLPS-IMRT). Xerostomia was assessed longitudinally at 3, 6, 12, 18 and 24 months based on the PARSPORT trial this is a phase III multi-centre randomised controlled trial of parotid sparing IMRT in patients with head and neck cancer. The efficacy of both treatments was similar, whereas the incidence of xerostomia was significantly lower in CSLPS-IMRT showing improvements in salivary function recovery and dry-mouth specifically increasing global quality of life score.

These last two studies only measured the severity of xerostomia reported by the patients in order to assess its influence on the quality of life of head and neck cancer patients after radiotherapy. There was no information related to the salivary flow rate (stimulated or unstimulated) and composition regarding dry mouth. In addition, systemic diseases, smoking and drinking habits were not measured, nor was there any information related to oral or dental assessment which could dramatically influence the quality of life of these patients.

Nutting et al., (2011) developed a multicentre study to compare side effects in 73 head and neck cancer patients using IMRT and conventional radiotherapy, in order to assess the incidence of severe xerostomia (grade 2) before starting the therapy and at week 4, 3 6 12 and 24 months, using Late Effects of Normal Tissue (LENT SOMA) scales. The European Organization for Research and Treatment of Cancer ‘s (EORTC) QLQC30 quality-of-life questionnaire was used and the associated head and neck specific module HN35. For the assessment of xerostomia, the modified xerostomia questionnaire was applied. Unstimulated and stimulated parotid saliva from each parotid gland were collected. After 12 months of RT, grade 2 xerostomia was significantly lower in the IMRT group than in the conventional radiotherapy group. At 24 months the same pattern of therapy was given and salivary flow rate, dry-mouth and global quality of life outcomes in this group improved. Despite the fact that they collected saliva and recorded the flow rate, the specific values showing the variation

in time were not given, only an expression of the results and whether the salivary flow rate was able to be measured or not at every time point.

Ghosh-Laskar et al., (2016), studied 59 patients with head and neck cancer treated with intensity modulated radiotherapy (n=30) or three-dimensional conformal radiation therapy (n=29). A clinical exam and salivary scintigraphy were performed, before and after radiotherapy. Grade 2 or worse acute xerostomia was evaluated at 8 weeks after radiotherapy, showing a significantly lower incidence in the IMRT group, the other acute toxicities not being significantly different. After radiotherapy, late radiation toxicity was measured every 6-months until complete 2 years. It was shown that grade 2 xerostomia in patients with HNSCC and treated with IMRT was reduced significantly in early (8 weeks) and late stages (24 months) after the treatment. Patients treated with 3D conformal RT showed an increased weight loss compared to IMRT group. In this study salivary flow rate was not measured clinically by sample collection. Therefore, there was no analysis of salivary biochemical composition after radiotherapy.

Chen et al., (2017) ran a retrospective study of 855 nasopharyngeal carcinoma (NPC) patients treated either with three-dimensional conformal radiation therapy (3DCRT) or with intensity-modulated radiation therapy (IMRT) between 2004 and 2009. Late toxicities were measured using Radiation Therapy Oncology Group (RTOG) criteria. Three years after radiotherapy, it was concluded that late toxicities occurred more frequently in patients treated with 3DCRT than in those treated with IMRT and salivary gland dysfunction was very similar in both groups. All the side effects were measured using the Radiation Therapy Oncology Group (RTOG) criteria. Salivary gland dysfunction was based on the observation of dry mouth and response to stimulation. Again, there was no report of clinical measurements or sample collection such as salivary flow rate and salivary composition in these two studies that compared the side effects of radiotherapy.

As a conclusion, after radiotherapy, head and neck cancer patients have a deteriorated quality of life related to xerostomia as reported by patients.

Dry mouth has been associated with a decreased quantity of saliva and as a result of altered protein composition (hyposalivation-independent dryness ranging from 4% to 50% in patients with dry mouth) (Närhi et al., 1994; Chaudhury et al., 2015, 2016). This subjective sensation

of oral dryness is a common side effect in head and neck cancer patients as well as salivary gland hypofunction caused by the cumulative dose > 20 Gy. An altered salivary production and quality due to radiotherapy may lead to changes in oral environment which in turn will Increase the risk of developing oral diseases, such as carious lesions and infections, along with reducing protective saliva functions such as hydration and lubrication of oral tissues (Chaudhury et al., 2015; Pedersen et al., 2018; Lynge Pedersen and Belstrøm, 2019).

Xerostomia affects the basic daily functions that are associated with quality of life of this group of patients producing discomfort when eating and swallowing and speech problems. Studies conducted are mainly based on patient reports after radiotherapy. Therefore, it is important to measure the variations in saliva production and salivary protein composition in order to correlate the results with xerostomia reported by patients before and after radiotherapy. Saliva protein composition has a direct influence on the normal hydration and protection of mucosal surfaces by its gel-forming capacities and lubrication properties. Altered salivary protein composition will change viscoelastic saliva properties leading to a reduced coating or wetness of mucosal surfaces. Specially mucins composition which are responsible of viscosity, elasticity and stickiness properties that influence directly in lubrication and mucosal covering. Therefore, not only the amount of saliva produced is important, but also protein composition is vital in keeping the integrity of the oral surface.

Finally, in the previous studies, there is a lack of information related with the subjects' habits (smoking and drinking), oral assessment in order to correlate xerostomia with the number of carious lesions and dental treatment received before and after radiotherapy.

1.5.4.5 Radiation Therapy Related to Salivary Protein Content in Unstimulated Whole Mouth Saliva in Head and Neck Cancer Patients

Radiotherapy produces major salivary gland hypofunction which leads to salivary flow reduction from the beginning of treatment and results in a long-term or permanent dry mouth, especially when the cumulative doses of radiotherapy are higher than 30 Grays

(Chao et al., 2001; Jawad et al., 2015). All salivary glands may be affected directly by radiotherapy during the cancer treatment. However, it has been well-established that serous acini cells are more radiosensitive than mucosal acini cells. Irradiation will cause cell death and inflammatory infiltration into the salivary glands leading to reduced saliva secretion and altered biochemical content (Jensen et al., 2003; SHAO et al., 2011; Hunter, 2013; de Paula et al., 2017).

Moreover, nasopharyngeal and oropharyngeal cancer radiation directly affects the acinar cells of parotid glands due to their proximity to the tumour, thereby receiving a higher dose of radiation which will damage cell membranes impeding their normal function (Dijkema et al., 2008).

After cancer treatment, rheological saliva properties will alter, becoming stickier and more viscous. It will also turn from transparent to white, yellow or brown (Jensen et al., 2003; Hunter, 2013; de Paula et al., 2017). Additionally, it has been stated that the concentration of salivary components depends on saliva flow rate. Thus, a diminished salivary gland function results in a reduced salivary flow rate. Therefore, saliva composition will be altered reducing its properties (Tschoppe et al., 2010; Dawes et al., 2015; Pinna et al., 2015; Gao et al., 2016; Riley et al., 2017).

It has been proposed that there may be a association between elevated caries prevalence, incidence and activity with salivary gland dysfunction (Dawes et al., 2015; Pinna et al., 2015; Gao et al., 2016). Specifically, the composition of proteins in saliva, their concentration and secretion rate are all responsible for oral health, maintaining saliva's rheological properties in order to maintain oral tissue integrity, function and oral homeostasis. Additionally, many other proteins are balancing the microbiome into symbiotic relationships, controlling opportunistic pathological bacterial/fungal growth through the power of altering

microorganism metabolism, increasing agglutination and clearance of particular bacteria species. Finally, these macromolecules are capable of maintaining optimal pH levels, reducing the acid moments and promoting the remineralization process over demineralization.

Salivary proteins, as a part of the enamel and mucosal pellicle, play an important role in biofilm development, promoting initial bacterial colonization and adhesion to all oral surfaces, modulating bacterial attachment, controlling its growth and metabolic activity. All of the above results in a healthy bacterial community, excluding pathogens or keeping these opportunistic microbes in a non-pathological state (Schenkels et al., 1995; Almståhl et al., 2001; Chiappin et al., 2007; Carpenter, 2013; Laheij et al., 2015; Dawes et al., 2015; Kilian et al., 2016). An increased concentration of IgA in saliva after RT has been related to mucosal damage and/or to cellular damage in the parotid gland (Richards et al., 2017).

Mucins form a protective, slimy and viscoelastic layer over the oral mucosa. It has been shown that mucin 5Bin particular is closely related to xerostomia in H&N cancer patients (Dijkema et al., 2012; H. L. Gibbins et al., 2014).

It is important to emphasise the protective effect of saliva on oral mucosa, which is not only related to the volume of saliva present in the oral cavity, but also to its quality. This helps to reduce the potential damage when a hot or cold drink is consumed, or from eating hard foods by lubricating them. This buffering capacity depends on the bicarbonate reaction and carbonic anhydrase VI action in converting carbonic acid into water and carbonic dioxide to neutralize acids (Dawes et al., 2015).

Salivary proteins are capable of forming a functional network, clustering different proteins. This network capacity will improve antibacterial, antifungal and antiviral properties, attracting beneficial microorganisms, protecting the oral environment from pathological bacteria and other harmful substances in order to preserve normal and beneficial flora which include around 700 species (Prodan et al., 2015).

Quality and quantity of proteins in saliva are associated with frequency and severity of oral diseases, indeed it has been suggested that many of these molecules are related to the aetiology of mucosal diseases, fungal infections and dental caries (Hemadi et al., 2017).

It has been established that a reduced flow rate in head and neck cancer patients treated with radiotherapy is linked to an increased caries risk and candida infections (Frenkel and Ribbeck, 2015).

Valdez et al., (1993)studied salivary flow rates from both unstimulated and stimulated saliva of the parotid and SM/SL glands, total protein content, lysozyme, lactoferrin, sodium, chloride, and potassium concentration. Fifty H&N cancer patients, with xerostomia after conventional radiotherapy, were compared with controls subjects. SFR in both stimulated and unstimulated saliva was reduced significantly compared with controls. Total protein concentration was similar in both groups, whereas secretion rate was lower in the irradiated patients. Lactoferrin was significantly elevated in parotid saliva as well as levels of lysozyme.

Funegård et al., (1994) analysed stimulated saliva from both parotid glands in 16 head and neck cancer patients before, during and post-RT for up to 18 months. They assessed α-amylase, hexosamine, sialic acid, salivary peroxidase, bacteria aggregating glycoprotein (BAGP) and IgA concentration in order to determine the antibacterial properties of saliva and correlate this with the diminished salivary flow rate. During the treatment, there was a protein concentration fluctuation which returned to baseline levels after 18 months. However, there were only 16 patients studied and parotid gland saliva does not represent whole mouth saliva.

It has been reported by Hannig et al., (2006) that total protein concentration did not show any variation in unstimulated whole mouth saliva of head and neck cancer patients compared to healthy subjects. It was reported that only the acidic proline-rich proteins were significantly lower in irradiated patients with hypo-salivation, following 6-12 months of radiotherapy. However, this group of ten patients showed variations in different salivary protein concentrations compared to healthy younger controls (34-44 years old). Without considerable differences, only with acidic PRP was a statistically significant decrease observed. Different proteins were assessed using a mass spectrometry with electrospray ionization (ESI-MS). There were no samples collected before cancer treatment and there was a lack of information regarding the oral health status of these groups of subjects.

Laheij et al., (2015) evaluated protein concentration using surface-enhanced laser desorption/ionization (SELDI-TOF-MS) in nine head and neck cancer patients treated with radiotherapy and ten healthy controls. Differences in concentration were found in antibacterial proteins, antifungal proteins related with oral infection, and proteins that maintain mineral homeostasis between both groups. Based on a small number of subjects with a wide age range, it was concluded that protein could be used as a biomarker of oral diseases related to RT in this group. Also, it was stated that oral health is associated with whole protein concentration, rather than a specific group of proteins. There was no systemic evaluation of the patients or medication intake. Moreover, an oral assessment could have been performed to correlate the results with existing carious lesions and a salivary protein composition assessment of the patients before radiotherapy.

Almståhl et al., (2001) compared the protein concentration in stimulated whole saliva from irradiated patients, Sjögren's Syndrome (SS) patients and those with hypo-salivation of unknown origin as well as healthy controls. The variation in protein in patients treated with radiotherapy was higher than the other hypo-salivation groups. The results were related with microbial species, salivary flow, periodontal disease and numbers of caries lesions found during the oral exam. Despite the fact that the groups were matched in terms of age, gender, number of teeth and periodontal characteristics, the number of subjects in the radiation group was half the number of the other groups. Furthermore, there was no information about the type of cancer, radiation dose received and habits of these patients. In addition, an oral assessment of these patients before their radiotherapy and treatments was not conducted. Consequently, further analysis is required to compare before and after RT and to follow up on the patients.

Eliasson et al., (2005) studied the secretion rate and IgA, albumin and lactoferrin in minor gland saliva and unstimulated and stimulated whole mouth saliva in 20 subjects with hypo-salivation due to Sjogren syndrome and radiotherapy compared with healthy, matched controls in gender, age and number of teeth. All patients were instructed to not clean their teeth inter-proximally for three days, not to brush their teeth the same day and not to eat or drink for at least 2h prior to the measurements. Flow rate from both minor and major salivary glands were diminished in patients with SS´s and the radiotherapy group compared with controls. All proteins in whole saliva were increased in comparison with controls; only

albumin was decreased in the SS´s group. Lactoferrin was significantly lower in the SS group compared with RT ($p < 0.05$). Minor salivary gland flow rate and secretion rate were diminished, and protein concentrations were higher in both groups compared with healthy controls. Additionally, the highest differences were observed in the RT group.

Vidotto et al., (2010) performed a proteomic analysis of saliva (n=7) and serum (n=15) from head and neck cancer patients before and after radiotherapy (between 1 to 60 months). Proteins that may contribute to tumour growth (palate lung and nasal epithelium clone protein PLUNC and zinc-alpha-2-glycoprotein) were studied and compared with control subjects. After radiotherapy, the protein profile analysed was similar to controls (n=29), showing the potential of saliva and serum for diagnosing and monitoring health, disease and treatment outcomes. Unstimulated whole mouth saliva is an accessible fluid composed of a mix of major and minor salivary glands and a mix of proteins that have different functions. Its composition and flux can be affected by several factors, including radiotherapy. The flow rate was significantly reduced after RT and differences in 13 protein profiles and their pattern of expression were shown between patients and controls (proteins involved in cell adhesion, cell differentiation and epidermis development, metabolic processes, transport and immune response). However, before and after radiotherapy, protein concentration did not show a significant variation. Flow rate and saliva constituents could be used to observe different pathological conditions. There was no hygiene assessment, record of number of months after RT and oral assessment.

Dijkema et al. (2012) studied mucin 5B (MUC 5B) concentration and total protein concentration (TPC) levels (mg/ml) in 29 head and neck cancer patients before and after 12 months of cancer treatment. After using a xerostomia questionnaire, the patients were grouped, according to their conditions, in non/mild and severe groups in order to correlate the amount of protein with the oral signs and symptoms of this side effect in differing degrees. Indeed, MUC5B concentration was higher in patients with no or mild xerostomia than in patients with severe xerostomia. Additionally, in this group, MUC5B output (mucin concentration by SFR) levels were the lowest but even so, there was no significant difference. Regarding total protein content, there was no statistically significant difference after the treatment among the groups. Two separate saliva samples were collected, stimulated submandibular gland saliva using citric acid solution (5%), collected by suction with a

micropipette in the floor of the mouth, in close proximity to the Wharton duct and parotid stimulated saliva using a Lashley cup. In addition, the xerostomia questionnaire used in the study was not described (Granger et al., 2007; Rohleder et al.,2009; Jensen et al., 2010; Papacosta et al., 2011).

Richards et al., (2017)assessed parotid function after 3 and 12 months of curative intensity-modulated radiotherapy (IMRT) in 26 HN cancer patients, analysing saliva´s organic and inorganic composition and flow rate. Subjective and objective xerostomia measures were also collected in order to associate this with saliva composition. UWM saliva flow rate decreased significantly along with total protein secretion rate, phosphate concentration and only lactoferrin was increased. The same trend was seen in SMS with lactoferrin secretion rate. At 12 months, lactoferrin was increased in both saliva samples. TPC secretion rate in UWMS was lower in the group with a high degree of xerostomia. This study was retrospectively planned and only included patients who had a determined flow rate >0.03ml/min.

In summary, despite the fact that all of these studies collected different types of saliva (parotid, sublingual, submandibular and unstimulated whole mouth saliva) from a small number of cancer patients (the majority of the studies recruited between 9 and 20 subjects), all attempted to correlate protein concentration with radiation oral sequelae such as carious lesions and oral mucositis. Some of the studies also correlated results with the number of S. Mutans and lactobacillus colonies, in order to identify proteins as a biomarker of bacterial oral diseases. Salivary gland function is altered after radiation therapy, affecting saliva flow rate and biochemical composition regarding protein concentration. The majority of these studies were performed after the radiation therapy. Hence, further longitudinal clinical studies are required to correlate the variation in saliva protein content with the most prevalent oral sequelae in head and neck cancer survivors. It is necessary to compare the total protein concentration with specific proteins related to antimicrobial, bacterial and sugar clearance, mucosal protection, lubrication, buffer and remineralization which are important salivary functions before and after cancer therapy, as well as age and gender-matched healthy controls, in order to identify potential protein associations and predict the oral and dental disease risk and finally provide a specialised individual dental care plan for each patient.

Finally, it would be interesting to associate the xerostomia induced by radiotherapy with the protein variation in saliva, especially the molecules that are part of mucosal pellicle.

It is important to establish the influence of salivary proteins on oral bacterial community as this will contribute to increase the pathogenic population and contribute among multiple factors such as diet, hygiene, mineral unbalance, to carious lesion onset. Thus, all the data obtained could be correlated in order to be able to predict the predisposition of caries development in this group.

1.5.4.6 Oral Mucositis

Oral mucosal inflammation produced by cancer therapy is one of the most common side effects in head and neck cancer therapy (Sonis, 2011; Epstein et al., 2012; Duarte et al., 2014; Franco et al., 2017; Jung et al., 2019). It is considered one of the most painful radiotherapy side effects strongly affecting quality of life of these patients (Franco et al., 2017).

More than two-thirds of radiation and chemotherapy-treated head and neck cancer patients suffer oral mucositis, presenting large, irregular and deep ulcers which reach the submucosa, due to the cytotoxic effects of inflammation on epithelial tissue (Sonis, 2004b, 2011; Epstein et al., 2012; Villa and Sonis, 2015; GB et al., 2015; Vasconcelos et al., 2016; Richards et al., 2017). As an acute effect, mucositis is influenced by tumour location, which is directly correlated to mucositis severity, cancer treatment (radiation dosage, size of the area and fractioning), genetic susceptibility and individual patient response, being described as a complex and multifactorial pathology. Additionally, other factors related with mucositis have been found, such as patients' habits. Smoking may modify the dose-response curve. Additionally, patients related risk factors such as a microbial population shift, dental and periodontal general condition, poor oral hygiene, reduced salivary flow rate and composition of saliva will influence negatively occurrence, intensity, severity and length of mucositis in head and neck cancer patients the possibility of oral mucositis onset (Sonis, 2004b; Epstein et al., 2012; Sonis, 2013; Villa and Sonis, 2015, 2016; Normando et al., 2017; Franco et al., 2017; Orlandi et al., 2018). Salivary mucins, especially MUC5B, are important in mucosal hydration and lubrication. Mucins are capable of forming a gel and covering oral epithelium, constituting

a dynamic and semipermeable barrier, in order to protect this tissue from dryness, shear stress and controlling bacterial adhesion and colonization. A healthy mucosal barrier is vital, particularly in patients with diminished salivary flow rate and altered biochemical components. Altered mucin composition will alter salivary viscoelastic properties resulting in a reduced or defective mucosal layer that will lead to a reduced hydration, lubrication, protection and diffusion of molecules into the oral epithelial surfaces (Larhed et al., 1998; Cone, 2009). Additionally, saliva contains a wide range of proteins which are vital in the different stages of wound healing (Brand et al., 2014; Dawes et al., 2015).

Oral mucositis is controlled by numerous molecules, inflammatory and non-inflammatory mediators, cytokines, growth factors and matrix metalloproteinases, such as epidermal growth factor (EGF) and tumour necrosis alpha (TNF-a) showing that this is not only an epithelium reaction to radiation, but rather a complex molecular chain of differing reactions(Villa and Sonis, 2016; Normando et al., 2017; Münstedt et al., 2019).

Such severe complications can be a limiting factor for cancer treatment, as the resultant pain of oral mucositis can cause patients to drop the treatment, compromising its effectiveness. More advanced cases will even require hospitalization to manage pain as well as parenteral feeding in order to endure the pain while feeding, with secondary infection treatment to prevent the risk of systemic infection, bacteraemia and sepsis. This is due to the higher risk of systemic infection among patients that develop mucositis. Thus, the prevention, management and control of oral mucositis is vital, so that the incidence and degree of this condition can be reduced (Epstein et al., 2012; Duarte et al., 2014; Franco et al., 2017; Almståhl et al., 2019; Münstedt et al., 2019).

There are five principle stages of mucositis progression. Initiation occurs in the basal epithelium and submucosa endothelium cells, despite the fact that the basal epithelium is the target of radiation toxicity and the site where cell injury and death start, by two mechanisms directly breaking DNA strands of cells and generating oxidative species. The radiation activates several pathways in endothelial fibroblasts and epithelium to initiate and modulate the damage response and tissue injury. Inducing immune cells (macrophages) to produce pro-inflammatory cytokines, such as tumour-necrosis factor α (TNF-α) and interleukin 6, lead epithelial basal cell death and injury (Sonis, 2004b; Vasconcelos et al., 2016).

Furthermore, these mechanisms of damage also harm epithelial stem cells, reducing their regeneration capacity, resulting in a permanently thinner epithelium barrier and allowing mucositis symptoms to emerge. Signal amplification occurs, with macrophages that are activated by radiotherapy producing pro-inflammatory cytokines in response, which amplify the primary signal, causing a positive feedback to magnify the tissue damage, by activation of metalloproteinases 1 and 3 (MMP-1 and MMP-3). After approximately 10 days of treatment, ulcerating lesions may appear in the oral mucosa (Sonis, 2004b, 2011).

Following this, spontaneous healing can begin 2 to 3 weeks after radiation, by migration of epithelial cells from the walls of the wound guided by proliferation, migration and differentiation signals from mesenchymal cells and extra cellular matrix (Sonis, 2004b).

Additionally, mucositis is associated with nutritional deficits, resulting in rapid weight loss, with the pain also presenting chronic fatigue apathy and depression. According to some reports, this could be linked to systemic serum elevated cytokines levels, that is a result of an increased mucosal proinflammatory cytokines production caused by mucositis (Silva et al., 2009; Franco et al., 2017; McCullough, 2017; Maria et al., 2017).

Certain studies have focused on regulating the pathways of oral mucositis, such as TNF-α, IL-6, IL-1β, IL-10, TGF-β, EGF, FGF, VEGF, specific tissue inhibitors and matrix metalloproteinases (MMP-2/TIMP-2 and MMP-9/TIMP-2 (GB et al., 2015; Normando et al., 2017). However, despite these mechanisms being explored, there are presently no therapeutic agents to prevent or rapidly resolve this oral side effect. Oral mucositis still remains as a dose limiting factor in head and neck cancer patient treatment, interfering with the plan (McCullough, 2017).

Finally, oral mucositis leads to a high risk of developing bacterial and fungal infections, with oral candida along with microorganisms capable of colonisation surrounding the ulcerated mucosa and contributing to the mucositis process, stimulating polymorphonuclear leukocytes (PMNs) and macrophages' chemotactic activities and pro inflammatory cytokines secretion. Ultimately, the bacterial colonies are capable of affecting the mucosal injury, severity and duration (Vanhoecke et al., 2015; Vasconcelos et al., 2016; Maria et al., 2017).

Clinical evaluation of oral mucositis related to Intensity Modulated Radiotherapy is not well established in the literature, possibly because there are several scoring criteria to determine

mucositis prevalence and intensity. Some studies only report the highest severity (3 or 4) leaving the lowest out of their scope(Logan et al., 2007; Epstein et al., 2012; Sonis, 2013; Villa and Sonis, 2015, 2016; Normando et al., 2017; Franco et al., 2017).

Oral mucositis diagnosis is based on clinical observation, with no scientific evidence of the superiority of one measuring criteria over the rest. These scales do not always meet the ideal criteria of being objective, sensitive, validated, reliable / easy to use, useful in different treatment modalities, asses mucosal damage and patients' daily function (De Sanctis et al., 2016).

In order to assess oral mucositis, the World Health Organization (WHO) developed an oral toxicity scale to quantify oral mucositis severity. It is based on a clinical observation of oral mucosa symptoms, signs of damage, and functional disturbances based on diet of the subjects (World Health Organization, Handbook, 1979). This scale is used in research and daily health care of head and neck cancer patients. The WHO scale grading ranges from no change to mild and including severe mucositis (grades 3–4). The training and experience in using the scale will improve the accuracy and consistency of the outcomes ('WHO handbook for reporting results of cancer treatment', 1979; Sonis, 2004a, 2013).

Oral mucositis monitoring is important, patients grade 3 or 4 are frequently reported because this group is more likely to delay their cancer treatment, reduce the radiotherapy dose (dose limiting toxicity), modifying fractionation or interrupting the cancer treatment continuity (approximately 30%), impairing the effectiveness of the therapy and affecting negatively the patients' quality of life. Due to the associated symptoms of severe oral mucositis over 60% of these patients will need parenteral nutrition, opioid analgesia, antibiotics and hospitalization so increasing the healthcare costs. Economic costs will increase as a result of prolonged cancer treatment and oral mucositis symptoms treatment(Sonis, 2004b; Maria et al., 2017).

Hahn Berg et al., (2004) investigated 127 head and neck cancer patients with advanced squamous cell carcinoma (SCC) treated with radiotherapy and at least 2 cycles of cisplatin and 5-FU. It was observed that 64% of patients developed mucositis, and 33% had grade 3–4 mucositis. In addition, other gastrointestinal symptoms were described that could be due to mucositis including severe nausea, dehydration and electrolyte imbalance which required intravenous or enteral fluid replacement.

Orlandi et al., (2018), assessed mucositis grades in a retrospective study in 132 head and neck cancer patients treated with IMRT (70Gy) and chemotherapy, in order to predict a dose response association to prevent and manage this side effect. Clinical parameters were assessed: age, gender, body mass index (BMI), smoke history, histology, staging, hypertension, diabetes mellitus, as well as cardiological, haematological and oncological diseases other than NPC, RT technique, fractionation and overall treatment time and chemotherapy regimens. There was no information regarding oral health and dental care received by the patients before and after the cancer treatment. Additionally, there was also a lack of information related to salivary gland function before and after radiotherapy, assessing salivary flow rate and saliva composition.

Jham et al., (2008) performed a retrospective oral assessment of cancer patients before, during and after RT to evaluate the oral side effects. Mucositis was the most prevalent side effect among subjects during RT, whereas after treatment completion it became the lowest in prevalence. Duarte et al., (2014)conducted a retrospective study of the prevalence of oral side effects in patients treated with IMRT and conventional RT. A complete oral evaluation was performed before treatment and patients with oral diseases were excluded. It was shown that there was a reduced number of mucositis and xerostomia cases among patients treated with IMRT compared with conventional RT. However, this group showed an increased number of caries lesions and fewer teeth remained in comparison to the other group.

In a retrospective analysis of oral side effects during and after IMRT,78 patients were seen every month during the first year, every 2 months in the second year and every 3–6 months after. Radiotherapy doses varied from 60 Gy to 70 Gy. Twenty patients received inductive chemotherapy and 35 received concomitant chemotherapy. Using RTOG index it was shown that the majority of patients (82%) developed severe mucositis during therapy (RTOG Grade 3 mucositis in 64 patients). Similar results were seen in 48 patients who had squamous cell cancer (SCC). It was shown that grade 3 mucositis was observed in 41 patients (85%). A median follow up was 18.7 months 34 patients presented xerostomia, 11 lost of taste, 7 neck fibrosis and 2 dysphagia (Van Gestel et al., 2011).

Similar findings were reported by Vera-Llonch et al., (2007) in a retrospective study in head and neck cancer patients treated with radiation therapy. The data was collected from 154 different oncologists, in order to determine the severity of oral mucositis. A non-validated

index was used, which categorised the patients in none, mild, moderate or severe mucositis. 83% of patients developed mucositis, presenting the following distribution: mild in 19%, moderate in 35% and severe 28%

It was also seen that patients who developed oral mucositis during treatment presented a higher risk of making unplanned breaks and hospitalizations, especially in the severe mucositis group. There is no record of any oral assessments or dental treatments given to these groups of patients before and after radiotherapy

All these studies are retrospective evaluations of the mucosal condition in head and neck cancer patients, reporting the prevalence and severity of oral mucositis during and after the radiation therapy. There is a lack of information regarding the oral condition of the subjects (dental and periodontal), dental treatment received before and after radiotherapy, oral hygiene, drinking and smoking habits and food consumption as well as sugar intake. Finally, there was no information related to salivary flow rate quantity and quality which are all related to the development of this condition.

Dodd et al.,(2003) studied the clinical efficacy of sucralfate (Carafate R) mouthwash for head and neck cancer patients (HNC) compared with salt & soda mouthwash in order to reduce the severity of the mucositis, the severity of mucositis-related pain and the time required to heal RT-induced mucositis. Habits like smoking and drinking were assessed. There were no significant differences in the number of days for mucositis to start, pain scores, grades and time of healing, during and one month after RT. The use of salt and soda was suggested because it is less expensive.

Veness et al., (2006) evaluated the effect of topical misoprostol in reducing the severity of radiation-induced mucositis in patients receiving a radical dose of radiotherapy in a randomized, double-blind, placebo-controlled trial. Forty-two patients received misoprostol and 41 received a placebo. No significant difference in the mean area under the mucositis curve (13.2 vs 16.6; P = 0.1) was noticed. The misoprostol group reported an increased soreness (7.6 vs 6.9; P = 0.04) and an increase in the use of analgesics. 12% of patients interrupted their treatment and the median time of interruption was 6 days. There was no difference between groups in the incidence of mucositis.

Diaz-Sanchez et al., (2015) evaluated the use of chlorhexidine gel 0.2% versus placebo in 7 patients diagnosed with head and neck cancer, in order to prevent and treat oral mucositis induced by radiation therapy and chemotherapy. Patients were instructed to apply the gel 5 times per day, from the initiation of radiotherapy until 2 weeks after the treatment finished. The grade of mucositis was measured using WHO score criteria, pain (visual analogue scale) and analgesic consumption was recorded by the patients, infectious complications were assessed in weekly reviews as well as treatment tolerance using a score from 0 to 5. After the treatment, mucositis pain and tolerance were similar in both groups and there was not any improvement with the use of chlorhexidine.

Franco et al., (2017) showed the impact of oral mucositis in twenty-one head and neck cancer patients' quality of life. All patients received intensity-modulated radiotherapy and they were using an oral mouthwash to prevent oral mucositis. A scale was used to assess the presence of ulcers and erythema in nine different places in the mouth. In order to appraise these patients' wellbeing, an oral mucositis questionnaire for head and neck cancer was applied weekly, along with a functional assessment of cancer therapy which includes physical, emotional, social, family and functional well-being. Additionally, pain was evaluated using a visual analogue scale, at rest and during daily functions (speaking and swallowing). It was shown that oral mucositis increases along with the increase in the radiotherapy dose. This was directly correlated with pain at rest, speaking and swallowing during the treatment and it was shown to have a negative impact on patients' quality of life in all the groups. This remained unchanged until two weeks after they finished their cancer treatment. This was a prospective study, there was no oral or dental assessment, salivary flow rate variation was not recorded, and composition was not assessed to correlate with dry mouth and taste acuity. Finally, regarding the impact of all these factors, together with the development of oral mucositis, it is also important to consider the influence of dietary advice and dental treatment received before and after radiotherapy in order to correlate with the data obtained.

Dodd et al., (2003); Veness et al., (2006); Diaz-Sanchez et al., (2015) conducted randomized controlled trials to evaluate the effectiveness of different medical products in preventing and treating oral mucositis provoked by oral cancer treatment. All the groups studied developed mucositis during the cancer therapy and there was no difference between the medication administered and the placebo group. Moreover, there was a lack of information about the

oral health status before the radiotherapy started, as well as salivary flow rate variation and protein composition of saliva.

Jham et al., (2009) assessed the effect of bethanechol administration in oral mucositis, candidiasis and taste loss before and during radiotherapy in 16 head and neck cancer patients randomly selected to use it. Mucositis was scored using the World Health Organization (WHO) method, candidiasis was diagnosed by clinical examination and taste loss was assessed by subjective report of absence of taste.

There were no significant differences observed between groups in relation to frequency and severity of mucositis, frequency of candidiasis and taste loss. Candidiasis during RT presented in a smaller number of cases in the group using bethanechol (50%) than the one using saliva (70%). The number of subjects that reported loss of taste was lower in the bethanechol group (69%) during RT, in comparison to the saliva group (95%) (P=0.07). It was seen that in this group, bethanechol does not seem to reduce the occurrence of mucositis, candidiasis nor taste loss during RT. In this study, there was no information regarding oral health, hygiene habits nor oral assessment including dental treatment received before and after the radiotherapy. Finally, there was no analysis of salivary flow rate nor composition which could affect the outcomes.

Charalambous et al., (2018)studied the efficacy of thyme honey in oral mucositis treatment to reduce symptoms, grade and secondary complications, such as weight loss and oral problems, in order to assess the effect of mucositis development and quality of life of patients. Seventy two head and neck cancer patients treated with IMRT (50 and 60Gy) were recruited and divided into two groups; intervention group (thyme honey rinses) and control group (saline rinses). Oral mucositis grade was assessed once per week starting at the 4th week of radiotherapy and until 6 months after, by radiation oncologists using a scale adapted by Radiation Therapy Oncology Group (RTOG). Quality of life was evaluated with the OM Questionnaire validated previously; a different questionnaire was considered to evaluate overall wellbeing. It was shown that in the intervention group, oral mucositis severity was less, patients maintained their weight and their overall health was better than control group increasing the Quality of Life index level in this group. However, the time it took to resolve lesions did not diminish in the intervention group compared with the control, taking between 2 to 4 weeks after finishing radiotherapy. Similarly, to the previous study, there was a lack of

information and of proper assessment of the oral health, dental health, salivary flow rate, saliva composition and dietary intake of these patients before, during and after radiotherapy. Finally, it is important to remark that honey was given three times a day for seven weeks, thus increasing the sugar content in these patients' diets. Also, the duration of every rinse was not given, and the oral hygiene habits were not assessed. All these factors may increase the risk of caries lesion development, which was also not assessed before and after radiotherapy.

Similar results were found in a study developed by Rao et al. (2017), where 49 head and neck cancer patients were divided into two groups, one was asked to rinse their mouth with poli-floral honey three times a day during and after the radiotherapy (mean dose received 62–70 Gy), the other group was using betadine (povidone-iodine) . In this study, the subjects from the groups were examined in order to assess decayed teeth and ulcers/lesions in the oral mucosa by an oro-dental physician. Oral hygiene habits were recorded, and all patients received oral and dental care during the radiotherapy. A senior oro-dental pathologist assessed the mucositis grade once per week, using the same scale RTOG index (Radiation Therapy Oncology Group grading system) until 4 weeks after radiotherapy. The results showed that honey was more effective in reducing oral mucositis, thereby decreasing the number of patients with intolerable mucositis. The number of patients that had to stop the treatment was reduced as well as the number of these patients, but there was there was no analysis of salivary flow rate and there is no information regarding the outcomes of the oral assessment.

Using RTOG index, Sun et al., (2019), studied the effect of two oral mouthwashes, one was pure vitamin B and the second was a mix of vitamin B and Gene Time VR (recombinant form of human epidermal growth factor capable of regulating cell-growth) to treat oral inflammation, in 100 head and neck cancer patients for 3 weeks after the radiotherapy (RT dose 50–70 Gy). The patients rinsed four times per day, 10 min after each meal and before going to bed, for a full five min and then swallowed slowly. The Radiation Therapy Oncology Group (RTOG) index was used to assess oral mucositis and the ulcer area was measured. The pain was recorded using a VAS scale. Almost all patients developed mucositis, but the mixed compounds group showed a significantly shortened ulcer healing time. There was no oral assessment or hygiene habits recorded. There was no evaluation of saliva quality and quantity either.

The main agreement in all studies including retrospective and prospective assessments, is that oral mucositis is a frequent and painful complication during cancer therapy; over 80% of oral cancer patients will develop this condition and it has been related to a reduced body mass index which will lead to dietary supplementation (Huang et al.,2019; Charalambus et al.,2018 ;Villa et al.,2016).

Presently, many of the studies are focused on finding an effective topical treatment and trying to reduce the incidence or severity of mucositis using a large number of natural and pharmacological agents. However, there is not enough evidence confirming the efficacy of these agents over placebo, in preventing and reducing oral mucositis development and severity related to radiotherapy (Riley et al., 2017 ;Huang et al., 2019).

In previously described retrospective studies, the prevalence of mucositis among groups of patients treated with radiotherapy was assessed. However, there was no clear measurement of xerostomia, salivary flow rate and saliva composition to associate with oral mucositis severity. It is important to consider both salivary flow rate and saliva biochemical content variation before and after radiotherapy to assess the role of both in oral mucosal health and pathology development. It is well established that dry mouth feeling is a result of a diminished saliva secretion. It has also been associated with a change in its biochemical composition without a diminished salivary flow rate (Wang et al., 2016; Riley et al., 2017). This protein association with salivary flow rate determines the variation in protein secretion rate after radiotherapy.

So far, there is no effective therapy to cure this side effect or reduce its severity and duration. Also, there are no drugs available to avoid or prevent radiation-induced mucositis, even though there are a large number of studies trying different products, such as promoting oral hygiene, food advice, reducing alcohol intake and quitting smoking. Recently, there have been studies focusing on antibody assessment, such as Interleukins 6 / 8 and TNF alpha, in HNC patients undergoing conventional radiotherapy to treat and prevent oral mucositis focusing on neutralizing the inflammatory response increasing defensive response (Villa and Sonis, 2016; Normando et al., 2017).

In summary, there is a lack of studies that analyse the protein content from unstimulated whole mouth saliva related to mucosal lubrication, hydration and protection in head and neck

cancer patients undergoing radiation therapy. Despite the fact that this is one of the most common complications of RT and dose limiting for HNC patients, usually causing intense pain, negative impact in QOL and a major economic and clinical impact along with the lack of effective drugs and treatments currently there is no biomarker to predict onset and severity of oral mucositis as well as early detection. Oral mucositis detection and diagnostic is limited to clinical manifestation of this side effect only.

Evaluation is required of protein concentration related to mucosal pellicle in head and neck cancer patients before/after radiotherapy. Comparing these proteins to determine the possible variation and correlation with the clinical information is needed in order to develop predictive “biomarker” signatures to identify patients with a high risk of developing severe oral mucositis.

Therefore, it would be important to develop a predictive model that could easily monitored and identify patients at risk of developing oral mucositis and severity, based on longitudinal quantification and identification of protein signatures present in UWMS associated with mucosal integrity in order to, help to improve prevention, management and increase overall treatment effectiveness.

Also, there is little research focused on oral microbiome variation before and after radiotherapy associated with secondary infection in oral mucositis, particularly using high throughput sequencing techniques to determine the microbiota present longitudinally that may be related to the severity of this condition.

Therefore, a prospective longitudinal study with a complete oral evaluation of head and neck cancer patients is necessary before, during and after cancer treatment which would help to identify and quantify clinical features that increase the risk of developing severe mucositis. There is a lack of information regarding salivary flow rate, protein secretion rate in order to correlate these factors with the onset and severity of oral mucositis.

Finally, it would be helpful to assess patients related factors that may modify the individual response to radiotherapy. An accurate oral assessment is necessary before starting radiotherapy, including evaluation of dental status (DMFT), oral hygiene, oral diseases and habits such as smoking and drinking.

1.5.4.7 Effect of Radiotherapy on the Interaction between Dental Organic Matrix and Apatite Crystals related to an Increased Caries Risk / Incidence

Patients with Head and Neck (H&N) cancer undergoing radiotherapy (RT) are at significantly higher risk of developing severe oral complications following treatment. These include rapidly progressive caries, which carry high risks of bone necrosis resulting in non-healing sites with extensive bone destruction should extractions be required. These are recognised as distinct disease entities related to H&N radiotherapy, termed “radiation caries” and “bone necrosis”, respectively.

(Vissink et al., 2003; Meurman and Grönroos, 2010; Walker et al., 2011; De Siqueira Mellara et al., 2014; Lieshout and Bots, 2014; Laheij et al., 2015).

Dental caries is the most common late side effect of radiation, however the nature of their origins or why the H&N patient cohort are particularly susceptible to them has not yet been fully established in literature. This susceptibility remains despite concomitant best preventative practice, comprising of frequent dental recall, regimes to improve acid resistance of teeth (0,05% sodium fluoride solution once per day, 5,000ppm fluoride toothpaste for use twice daily), pre-radiotherapy assessment performed by the dental team in order to identify pre-existing diseases and potential risk during and after RT, and preparing the patient for the possible side effects of cancer treatment. In addition, this increased susceptibility may persist throughout a H&N cancer patient’s lifetime, leading to a complete edentulousness in the years following the cancer treatment and affecting their quality of life.(Vissink et al., 2003; Meurman and Grönroos, 2010; Walker et al., 2011; De Siqueira Mellara et al., 2014; Lieshout and Bots, 2014; Laheij et al., 2015).

Typically, dental caries are present a few months after radiotherapy, with a rapid onset as a result of a combination of factors including shifts in oral microbial community composition favouring acidophilic bacteria, poor oral hygiene due to potential mucositis, hypo-salivation, altered salivary composition and dietary alterations such as an increased sugar intake during and after the cancer treatment for weight maintenance (Springer et al., 2005; Kielbassa et al., 2006; De Oliveira Mota et al., 2013; De Siqueira Mellara et al., 2014; Gonçalves et al., 2014; Pinna et al., 2015; Lu et al., 2019).

Histological analysis has revealed that the onset and development of post-radiation caries are similar to non-radiation-related caries, but the sites affected are different: radiation-related caries are more frequently located on the cervico-labial surface, incisal edge of canines and incisors (Walker et al., 2011) whereas non-radiated caries are more frequent in the interproximal and occlusal areas of the posterior teeth (molar and premolars) (Laheij et al., 2015; Lu et al., 2019).

Further patients with radiation-related caries often have little or no pain, even in the most severe cases(Walker et al., 2011). The clinical appearance and patterns of the radiation-related caries are often different and typically observed as brown discoloration of the enamel on irradiated subjects. Although the reasons for such differences is not well-established, it has been suggested that this could be considered a sign that radiotherapy-related caries are uncavitated and should be treated as an incipient caries (Silva et al., 2009; Lu et al., 2019).

First instances of radiation-related caries are characterised by localised lesions to the incisal or occlusal surfaces. Their development continues with a partial or total enamel delamination, which diffuses and progresses by developing an irregular and generalized erosion of the incisal edge of the tooth, ultimately resulting in dentine exposure and leaving tissues vulnerable to decay (Gomes-Silva et al., 2017). Furthermore, lesions with a half-moon demineralization pattern with rapid progression can be seen in root surfaces after radiation therapy, which may lead to tooth fracture (Silva et al., 2009).

The delamination observed post-RT has been associated with a biomechanical breakdown of the dentinoenamel junction (DEJ) caused by radiation (De Siqueira Mellara et al., 2014; Lu et al., 2019). RT induces further changes in the organic composition (collagen) and increasing organic matrix degradation (MMPs), resulting in disruption of the DEJ area. In healthy patients, this area is not typically affected by mechanical failure and even with parafunctional loading, it is capable of impeding crack propagation. The DEJ keeps enamel and the underlying dentin firmly joined by collagen fibres (type I, IV and VII), allowing for increased stability, fracture resistance and a capacity to absorb energy and plastically deformation without fracturing (J. D. McGuire, Gorski, et al., 2014; Jacob D. McGuire, Walker, Mousa, et al., 2014; Jacob D. McGuire, Walker, Dusevich, et al., 2014).

The mechanisms that control enamel delamination and debilitated mechanical properties after RT have been explored in vitro, with results suggesting that RT alters mechanical properties, such as increasing elastic modulus and stiffening within the tooth structure(Kielbassa et al., 2000). RT also alters enamel microstructural organisation and protein / mineral compositions that affect normal teeth functions., This increases tooth fragility and susceptibility to fractures. Clinically, this would lead to rapid tooth destruction under physiological loading, increasing the wear of the incisal and occlusal areas during mastication and may ultimately lead to fracture of the crown (Santos-Silva et al., 2015).

These aggressive types of caries represent one of the most severe consequences of RT for H&N cancer patients, with potential risks of complete edentulousness within 3 - 5 years after RT that has a severe impact on the patients' quality of life (Jansma et al., 1989; Kielbassa et al., 1999, 2006; Walker et al., 2011; Lieshout and Bots, 2014).

Goncalves et al. (2014) and Fränzel and Gerlach, (2009) analysed the mechanical properties of irradiated molars, before, during and after RT. Fränzel and Gerlach, (2009) reported a significant reduction in hardness and elastic modulus in the enamel related to the RT dose, whereas Gonçalves et al., (2014) found an increased micro-hardness in the enamel surface after radiation. In line with finding by Reed et al., (2015) both studies reported a reduction in dentine micro-hardness. They observed that the elastic modulus and stiffness were increased in the enamel and dentine near the enamel dentine junction (EDJ) after RT. In addition, Raman spectroscopy showed that the protein/mineral ratio was significantly reduced in the whole enamel and superficial dentine towards the EDJ which could contribute to the enamel delamination that happens after the RT. However, the study sample number was only 7 teeth. This sadly is too few to draw definitive conclusions. Further the fractioning protocol was higher than the one used in treatment of H&N cancer patients.

It has been suggested that irradiation itself does not affect micro-hardness in demineralized enamel, rather the oral hygiene habits of the study subjects affected the enamel properties indirectly A difference in mineral loss was observed in the two studies which has been attributed to the brushing habits of the five participants in the first study and the 12 participants in the second study, who were wearing the appliances with irradiated and non-irradiated enamel slabs. It is important to state that the participants had similar oral conditions, caries risk, treatments and general health. Also, unstimulated whole mouth saliva

(UWMS) flow rate was measured with all participants using a fluoride-free toothpaste, but the technique and frequency of brushing was not described. On the other hand, the teeth were kept wet during radiation (Kielbassa et al., 1999, 2000).

However, subsequent studies have shown that RT reduced the tensile strength (UTS) in enamel and dentine, with and without the use of specific mouthwash. It is important to remark that the teeth were kept in artificial saliva during radiation (Soares et al., 2010; Soares, Roscoe, et al., 2011). Afterwards, the same group irradiated (60Gy) premolars divided in sound and endodontically treated and restored with resin or amalgam (mesio-occlusal-distal preparation) and compared fracture resistance and strain of cusp with non-irradiated premolars. It was observed that fracture resistance was significantly lower in sound irradiated premolars; also strain of cusps were significantly higher than the non-irradiated sound premolars (Soares, Roscoe, et al., 2011).

Radiation caries is the main cause of tooth destruction in this specific population (Vissink et al., 2003). It may directly affect the chemical/molecular tissue content by altering the hydroxyapatite crystals in the prismatic structure and collagen fibres near the EDJ. These molecular changes may affect micro-hardness, stiffness and fracture resistance of teeth, facilitating caries development (Kielbassa et al., 1999; Soares, Neiva, et al., 2011; Soares, Roscoe, et al., 2011;De Siqueira Mellara et al., 2014; J. D. McGuire, Mousa, et al., 2014).

Qualitative analyses of the irradiated teeth using different microscopy techniques to describe the caries zone and fracture patterns after loading, respectively have been performed (Kielbassa et al., 2000; Soares et al., 2010; Soares, Neiva, et al., 2011; De Oliveira Mota et al., 2013). However, these studies give an anatomical description only (qualitative analysis) and there is a lack of information regarding the protein concentration, mineral molecular composition and chemical variation of irradiated hard tissues. . McGuire, Mousa, et al., (2014) investigated the protein composition in the EDJ in healthy teeth, concluding that the enamel near the EDJ contains collagen IV and VII as well as laminin. Collagen IV provides a molecular link between enamel and dentine, so the loss of this protein due to RT may produce instability in the EDJ, facilitating post-radiation enamel delamination. More recently, the same group (J. D. McGuire, Gorski, et al., 2014) performed an in-vitro assessment of collagen IV in non-irradiated and irradiated teeth, using the same method of collagen detection, identifying this protein as a biomarker of the enamel dentine junction (EDJ) in mature teeth. In this study,

irradiated teeth showed a reduced staining pattern of this protein at the EDJ, indicating that *in vivo* radiation may possibly affect collagen IV. Conversely, Springer et al., (2005) did not find any difference in collagen fibre concentration in hard tissues of non-irradiated and irradiated teeth. Only the pulp tissue showed a shift in protein concentration after RT, which may affect odontoblast metabolism and impair the capacity of these cells, thus reducing their capacity to produce reactionary and reparative dentin as a barrier protecting them from injury. However, this study only involved samples of teeth irradiated in vitro, without any influences from the oral environment. Further studies are required to determine whether the time elapsed since the treatment has any influence on these protein changes.

In the same pattern of McGuire et al, Hui lu et al. (2019) recently compared *in vitro* micro hardness and elastic modulus of 60 sound third molars divided into three groups, one non-irradiated (control) and the other two groups received 30 and 60 Gy respectively. These specimens were exposed to a 2 Gy fraction per day, five days per week in order to reproduce the clinical treatment conditions and controls were kept in saline solution. Additionally, the microstructure was assessed using a scanning electron microscope (SEM) and the mineral protein ratio was determined with a Raman spectroscopy. After RT, there was a significantly decreased micro hardness close to the EDJ in both irradiated groups compared with the non-irradiated. Fractures and fissures were present in the vicinity of EDJ in the irradiated groups. Elastic modulus of the irradiated groups followed a similar trend in the EDJ zone. SEM revealed a destruction of the enamel structure related to the dose of radiation. Cracks, irregular prisms and degeneration of the collagen network were seen in both irradiated groups. In contrast, protein phosphate ratio did not vary significantly in the irradiated groups in enamel, but in dentin the protein mineral ratio dropped in direct correlation with the radiation dose. Even though this is one of the few studies that analysed the possible mineral protein composition changes in the EDJ region after RT, the RT protocol for the patient cohort has not been clearly described in the study. Further necessary oral conditions during the radiation delivery were not reproduced such as facial hard and soft structure, presence of saliva (the teeth were dry), bacterial composition, food intake and oral hygiene. It is also important to take into consideration the cancer location and cancer staging to assess the size and location of the primary tumour (T), lymph nodes (N), and the possible distant metastasis (M).

This tumour stage evaluation relates to the prognosis, determine the area and size of the radiation beam and lastly the real amount of radiation delivered on these molars, which today may be outlined with specific computational programs.

Qing et al., (2015)analysed the effects of RT on the wear resistance of enamel on 19 teeth collected from orthodontic patients and cut in slides which were perpendicular and parallel to enamel rods. Enamel specimens received 60 Grey fractioned 2Grey per day, 5 days a week; samples were immersed in cups with artificial saliva and changed daily. Nano scratch tests were conducted; X-Ray diffraction to evaluate the crystal structure of the enamel before and after radiation and FTIR spectrometric analysis was performed to obtain spectra of the enamel before and after RT. In addition, scratch resistance and micro hardness were analysed before and after RT. A modification of the crystallography and composition of the enamel was found after irradiation which would lead to a reduced wear resistance of enamel, indicating inferior mechanical properties. This is a possible explanation of the enamel delamination observed clinically after RT in H&N cancer patients. Although in this study they tried to imitate the oral condition, by keeping the teeth in artificial saliva, the sample number was lowered, and the focus was on enamel properties cutting the teeth which is not a representation of real-life clinical situations.

To date, no definitive conclusion may be reached from the studies related to the direct effects of radiation on the mechanical properties of teeth. The reported findings indicate a decreased surface, micro hardness and ultimate tensile strength, as well as an increased stiffness and brittleness of enamel, but most of the studies irradiated in vitro extracted teeth which are not the conditions faced by H&N cancer patients during and after treatment. These studies also used a small sample size with radiation being applied directly to the teeth. The final radiation dose and fractioning are not the same among the different studies. Also, the type of teeth, storage time and conditions, during and between RT doses, are not consistent with some studies ensuring the teeth are kept wet and others not. The participants' age is another important factor to consider as this will affect the chemical composition of teeth. Additionally, there are only a few studies that have been investigating the mechanical properties of the EDJ proximity which plays an important role in fissure propagation. This is important given that the RT caries process starts with enamel shear fracture leading to a complete delamination from dentine.

Therefore, it is necessary to conduct studies on living teeth, imitating the clinical condition of the mouth with accurate radiation doses being delivered to larger sample sizes, in order to produce data that is directly translatable to the clinical practice. Additionally, quantitative image analysis of dentine obtained by two-photon microscopy (Second harmonic generation; SHG) would enable the characterisation of the collagen fibre structure (Sun et al., 2006; Kim et al., 2000). It would therefore be possible to gather information about size of collagen fibres, their interaction with mineral components, degree of organisation and cross-linking (Stoller et al., 2002). High resolution two-photon fluorescence microscopies would allow for the non-destructive observation of tissue in 3D (z-scan mode enabling optical sectioning & subsequent 3D reconstruction). The mineral content of dentine samples may also be analysed by Raman spectroscopy (Atmeh et al., 2015).

RT has been associated with decreased quantities of bacterial colonies in the dental biofilm, associated with non-cavitated stage of caries lesion non mutans streptococci (S. sanguinis, S. oralis, and S. mitis) and Actinomyces, which play an important role in keeping the stability of the microbial ecosystem in order to maintain the balance between demineralisation and remineralisation of the tooth surface (mineral gain vs mineral loss) (Takahashi and Nyvad, 2011). It has been observed in children with early childhood caries (ECC) that candida spp. are capable of invading dentinal tubules, secreting acid and helping enamel demineralization; They are also capable of binding hydroxyapatite to dissolve crystals. In this group of children, this microorganism was relevant in caries progression (Belstrøm et al., 2016; Hemadi et al., 2017).

However, further studies that explore the clinical in vivo results are not fully established, with past studies exploring the direct impact of radiation on teeth damage and the ensuing micromorphological changes that result in enamel delamination and finally in aggressive caries formation (Gomes-Silva et al., 2017).

In addition, the majority of the studies performed so far showed a lack of information about participants in relation to systemic diseases, medication, oral diseases, caries risk, caries lesion number, localization, teeth/surface and dental treatment. Saliva flow rate and composition were not taken into account in these studies, even though a diminished saliva flow rate might accelerates the progression of dental caries considerably (Takahashi and Nyvad, 2008; Kilian et al., 2016; Belstrøm et al., 2016). By performing an oral clinical

assessment along with the described measurements, it is possible to draw a holistic clinical picture of H&N cancer patients pre and post RT and to compare them with healthy matched patients, using internationally recognised metrics and indices of oral health (e.g. Decay Missed Filled Tooth (Surface), International Caries Detection and Assessment System (ICDAS) Periodontal probe (CPITN), gingival index and plaque index).

1.5.4.8 Quality of Life

As discussed previously, the patients' nutritional intake, food preferences, social communication skills and overall mouth feel deteriorate following RT. Therefore, such patients often require a multidisciplinary team approach (restorative dentists, periodontology, endodontic, dental hygienists, radiation oncologists, medical oncologists, and oral surgeons) to maintain oral health, recover functional problems and improve quality of life (QoL) (Meurman and Grönroos, 2010; Epstein et al., 2012; Jawad et al., 2015; Murphy and Deng, 2015). This can involve a process of continuous recall consultations for monitoring purposes, following RT treatment, in order to analyse the risk of tooth breakdown, which becomes more severe with time (Meurman and Grönroos, 2010).

In addition, there is an economic cost involved with follow-up care, as ancillary support teams of nutritionists, dietary counselling and speech therapy are required to restore a patient's QoL, following cancer treatment in order to prevent depression (Wissinger et al., 2014). As almost 50% of cancer survivors report clinical depression or stress, in relation to xerostomia and their increased DMFT, which affects their ability to eat in public and has a great impact on their QoL (Duke et al., 2005; Osailan et al., 2012; Rieger et al., 2012; Lieshout and Bots, 2014; Deng et al., 2015).

1.6 Intensity-Modulated Radiotherapy

In order to try to diminish the adverse side effects of the RT, three-dimensional computed tomography planned radiotherapy known as "Intensity-Modulated Radiotherapy" (IMRT) has been introduced (Pacholke et al., 2005; Pow et al., 2006; Vergeer et al., 2009; Porter et al., 2010; Murphy and Deng, 2015; Jawad et al., 2015). IMRT permits the sparing of healthy tissues that are near tumours, specifically salivary glands (parotid) in order to reduce the risk of hypofunction. Unlike conventional radiotherapy, in IMRT the radiation dose is delivered

with more precision and also different intensities can be delivered to the tumour. IMRT uses seven different beams, each with a regulated strength, shaping different doses and permitting better targeted volumes. However, it is not always possible to avoid toxicity from radiation of the normal adjacent tissues (Dawes et al., 2015; Brennan et al., 2017; Strojan et al., 2017). IMRT reduces the radiation dose on parotid glands, eyes and the cervical spinal cord, while at the same time maintaining clinical effectiveness to reduce the risk of under treatment of nearby areas of microscopic tumour spread. It is important to design an accurate target volume, keeping the dose distribution only on the tumour and sparing the normal structures reducing salivary gland damage (Vergeer et al., 2009; Porter et al., 2010; Randall et al., 2013; Duarte et al., 2014; Brennan et al., 2017).

In particular, it is well established that parotid gland salivary flow decreases after conventional radiotherapy, in relation to the mean dose volume irradiated. However, there have been studies demonstrating how salivary flow rate can be improved and xerostomia reduced in cancer therapy, with the adoption of IMRT improving the quality of life of these patients (Little et al., 2012; Wang and Eisbruch, 2016; Brennan et al., 2017; Scrimger et al., 2018). Unfortunately, the causalities of the improvements and variations in biochemical composition of saliva after IMRT are still not clear (Randall et al., 2013; Brennan et al., 2017), therefore it is necessary to quantitatively analyse the saliva components, proteins and microbial changes that occur in patients' teeth/oral cavity following IMRT, in order to determine the aetiology and pathophysiology of the two prevalent side effects (caries/mucositis) to correlate with preventive measurement and appropriate treatment for these patients. Finally, it is also necessary to perform a complete oral assessment using validated indices in order to create a baseline record before treatment and a validated measure to compare against following IMRT and with healthy individuals.

1.7 Saliva as A Multicomponent Fluid for Early Clinical Diagnostic of Disorders

A biomarker is a biological molecule, biochemical component, gene, cell or bacteria that is an indicator of pathologic or physiologic conditions and pharmaceutical response to treatment. It must be able to be measured in order to predict, diagnose and assess the treatment of a disease. Additionally, it should have a high sensitivity, specificity, functionality, low cost and the capacity of testing a high number of samples in order to optimize laboratory services. A

predictive biomarker is related to clinical outcome and is measured before treatment. It will provide information regarding progression of a disease given certain treatment. Prognostic biomarkers provide information of disease development without considering any specific treatment (Wong, 2011;Gao et al., 2016; Wang et al., 2016; Pedersen et al., 2018). It is well stablished that saliva biochemical composition is responsible for multiple functions (Carpenter, 2013a; Pedersen et al., 2018; Lynge Pedersen and Belstrøm, 2019). A broad range of saliva components may help to identify systemic conditions or detect the exposure to dangerous substances. These elements represent potential biomarkers to detect different diseases including oral squamous cell carcinoma (OSCC), autoimmune diseases, viral diseases, bacterial diseases, cardiovascular diseases, and HIV among other conditions. Saliva secretion rate and composition have been used to assess salivary gland function as a biomarker of hypofunction when the flow rate was reduced. Saliva biochemical constituents (proteins and peptides) have potential for diagnosis, monitoring the course of oral diseases and treatment evaluation. In addition, saliva contains a mix of products from the oral tissues affected by caries or periodontitis which is why could be used to monitoring diseases progression and treatment success rate (Chaudhury et al., 2015; Proctor, 2016; Zhang et al., 2016Pedersen et al., 2018). Saliva proteins are relevant in tooth protection, preventing demineralization and increasing remineralization, others participate in defence having antimicrobial properties or promoting certain bacterial colonization during biofilm formation. Protein constituents of saliva that are capable of regulate oral microbial composition and host defence have been tested as early childhood caries markers. Assessing these proteins as a potential indicator of dental health in children, provide information to predict caries risk and prognosis (Hemadi et al., 2017). Additionally, proteins such as cystatin S have been assessed for a potential marker of submandibular gland dysfunction in SS patients. It was stated in a study including only five patients diagnosed with syndromes, the possible correlation between cystatin S and pathological findings in submandibular gland (UWMS salivary flow rate) (Martini et al., 2017).

Vitorino et al., (2005)studied the association between salivary protein composition and dental caries in 20 males, divided according to DMFTS index into caries-free and caries-susceptible. From the caries-free group, 6 subjects were selected to carry over 2 hours, pieces of sterilised enamel bonded to their molars. Afterwards, enamel pellicle was collected from the pieces and analysed. An increased amount of peptide fragments (sign of proteolytic activity) and

lower amounts of proline rich proteins, histatins and statherin were found in caries-susceptible subjects, which could increase their risk of developing caries.

Regarding periodontal disease presence/severity and therapy response, several studies have compared different salivary protein concentrations and specific oral pathogens present in patients with chronic periodontitis compared with healthy control subjects.

On one hand, there are studies focused in biochemical salivary biomarkers to assess treatment response in adults with chronic periodontitis. These studies were focused in salivary immunoglobulin concentration such as IL-1b and matrix metalloproteinases (MMP-8) connected with extracellular matrix degradation to screen periodontal disease severity and treatment. Combining host inflammatory response and molecules related with tissue damage signs (Miller et al., 2006; Ebersole et al., 2015). On the other hand, there are studies analysing periodontal pathogens associated with periodontal disease progression in order to improve periodontal treatment. The number and ratio of porphyromonas gingivalis, Prevotella intermedia and Tannerella forsythia present in stimulated saliva analysed from 85 patients during 18 months were found significantly correlated with presence of periodontitis having the potential of predicting the progression of this disease (Nomura et al., 2012).

Lee et al., (2012) studied 30 salivary proteins related to host defences, inflammatory process and tissue destruction were analysed and associated with the proportion of bacteria present in subgingival plaque to assess gingivitis treatment, in 30 healthy subjects. Unstimulated whole mouth saliva was collected during and after the treatment of the provoked gingival disease. Increased levels of IL-6 and MMP-1 at baseline were associated with risk of developing a higher inflammatory response.

Miller et al., (2006) analysed possible biomarkers for three aspects of periodontitis inflammation, collagen degradation and bone turnover associating these with clinical characteristics of periodontal disease. Unstimulated whole mouth saliva was collected from 28 subjects. Salivary levels of MMP-8 and IL-1β appeared to serve as biomarkers of periodontitis.

Potential salivary biomarkers are being tested for identification and monitoring diseases including periodontitis, dental caries, oral cancer, but also systemic diseases like Sjögren's syndrome (Pedersen et al., 2018).

Unstimulated whole mouth saliva collection presents many advantages such as being minimally invasive, accessible, minimum cost, reduced anxiety and increased comfort for patients during collection. Moreover, unstimulated whole mouth saliva collection does not require special training or equipment to obtain samples. In addition is more accurate than stimulated saliva because the agents used to stimulate the salivary flow may change salivary composition (Lee et al., 2007; Vidotto et al., 2010; Franzmann et al., 2012; Chaudhury et al., 2015). It is important to standardize the salivary sample collection to avoid circadian variations in salivary flow rate and composition (Dawes and Ong, 1973). During and after collection saliva samples must being kept on ice at 4C°, aliquoted and frozen immediately after collection to avoid proteolysis and deglycosylation of the proteins (Takehara et al., 2013).

Nowadays personalized medicine is becoming an area of interest in research in order to create a tailored treatment according to patients' personal characteristics such age, bodyweight, body mass, gender, renal and hepatic function, local oral factors and genetics is the main goal of medicine in order to avoid severe and potentially life threatening drug toxicity for patients (Zhang et al., 2016).

Novel ways to detect oral and systemic diseases are developing rapidly, including proteomics, genomics and molecular biological methods, that can be used to assess salivary protein components. Saliva can contain substances present in human serum due to a thin layer separating salivary ducts from systemic circulation enabling the exchange through the capillary walls into the salivary glands duct. In addition, saliva contains specific proteins that can be used also as a marker of different systemic diseases, instead of multiple test that are more invasive, expensive or in many cases non-conclusive (Osman et al., 2012).

The discovery and use of molecular biomarkers in saliva associated with oral diseases would permit fast and precise diagnoses and possibly more accurate treatment as well as controlling of disease activity.

Mucositis severity and frequency is related to cancer therapy, volume of irradiated mucosa, fractionation regime, dose per fraction and total dose. However, there are some patients with similar malignancies and treatment regimens results in different mucositis severities. Therefore, it is important to identify specific risk / predictive factors related to patients

comprising age, body weight, body mass, gender, renal and hepatic function, use of alcohol and tobacco, genetics and oral health factors (Rosenblatt 2014) Among oral factors is important to consider salivary flow rate, salivary composition and oral bacteria which will influence oral mucositis development (Sonis, 2011; Villa and Sonis, 2015).

It is important to comment that around 20% of oral mucositis patients will have a reduced 5-year survival rate due to the subsequent cancer treatment disruptions. Which is why research should be focused on prevention and treatment in order to reduce its prevalence and severity (McCullough, 2017).

Unfortunately, prediction of onset and severity of oral mucositis is not possible yet, an early identification of high-risk patients through predictive markers would allow to create a specific monitoring regime and characterization of oral mucositis in order to prevent, prepare, reduce its severity and elimination.

Certain molecules have been assessed to evaluate severity and progression of oral side effects of cancer therapy, including citrulline, calprotectin and the pro-inflammatory cytokines (Al-Dasooqi et al., 2013).

However, there is no definitive way of measuring the risk of developing oral mucositis before radiotherapy and predict severity to improve the management of such condition or prevent severe toxicities (Normando et al., 2017).

In this way, it is imperative to analyse salivary proteins variation post IMR and evaluate their associations with oral mucositis assessment in order to find a possible predictor of onset and severity of this side effect to help to develop patient customized intervention, to prevent the development of the condition or severe stages (Sonis, 2011).

Chapter 2 Clinical Assessments in Head and Neck cancer patients

2.1 Introduction

Radiotherapy will produce a diverse range of side effects many of which are oral complications due to the proximity of the tumour to the salivary glands (Tschoppe et al., 2010; Nutting et al., 2011; Richards et al., 2017). These are interconnected with salivary gland hypofunction inducing a reduction in salivary flow rate, leading to oral side effects (Wijers et al., 2002; Nutting et al., 2011; Belstrøm et al., 2016J; anus et al., 2017), despite the current support from a multidisciplinary medical and dental team pre- and post-cancer treatment and the use of IMRT (Walker et al., 2011; De Siqueira Mellara et al., 2014; Lieshout and Bots, 2014; Laheij et al., 2015). It has been reported the occurrence of xerostomia and taste altered perception (Ruo Redda and Allis, 2006; Epstein and Barasch, 2010; Epstein et al., 2016; Spotten et al., 2016; Epstein et al., 2019) are linked to reduced salivary flow rate following HNC treatment (Epstein et al., 2019). Moreover, a reduced flow rate is usually accompanied by an altered composition, leading to an increased risk of oral diseases, as well as hastening the progression of dental caries and mucosal injuries along with bacterial colonization. It is well established that saliva is important to maintain tooth and mucosal integrity, throughout its protective functions (Hannig et al., 2017; Pitts et al., 2017).

Oral mucositis has a significant negative impact in HNC patients care, altering their cancer treatment course, reducing quality of life and increasing the therapy cost which is why it is important to report its incidence and severity along with an analysis of the possible link with other side effects to reduce its severity because there is no effective treatment or preventive therapy. In addition, mucositis and its complications are often under reported due to the wide range of scoring criteria used to determine mucositis prevalence and intensity. This represents a major drawback in this research field, as the lack of an objective reliable and valid index to compare different clinical reporting and the fact that some studies only report the highest severity grades (only 3 or 4) leaving the lowest out of their scope, can underestimate the overall prevalence of mucositis. Therefore, is not easy to draw conclusions regarding the clinical evaluation of oral mucositis related to IMRT (Logan et al., 2007; Epstein et al., 2012; Villa and Sonis, 2015; Normando et al., 2017; Franco et al., 2017).

The clinical management of these deleterious RT side effects represent a challenge for the care team and there is a necessity to develop optimal preventive and therapeutic regimes. Clinicians need to understand the consequences associated to cancer treatment in order to develop preventive care plans and treatments for acute and chronic side effects in order to improve HNC patient's quality of life. The first step is to collect data and understand the mechanisms; a clinical oral assessment pre- IMRT (T0) and post radiotherapy at 6 months (T1) and 12 months (T2) in order to determinate a baseline to compare over time. The second part of this analysis was assessing the possible association among the dental and oral evaluation outcomes post IMRT.

2.2 Aims

The aim of this longitudinal trial was to assess salivary gland function and oral/ dental status of HNC patients pre (T0) and post IMRT (T1 and 2) and analyse the possible associations between the clinical outcomes.

Null hypothesis: IMRT will not affect salivary gland functionality and oral condition.

Objectives

- To quantify the variations in flow rate of UWMS at T0 and compared to T1 and T2 in ml per minute.
- To assess the effect of IMRT on teeth in HNC patients at different time points, and furthermore to associate those clinical assessments with salivary flow rate.
- To assess xerostomia, utilising subjective dryness perception of patients at T1 and T2, whilst also determining whether hyposalivation has occurred in accordance to self-reported xerostomia by patients.
- To assess subjective taste alteration utilising questionnaire following IMRT, whilst also determining whether hyposalivation has occurred in accordance to self-reported taste changes by patients.
- To report clinical oral mucositis presence and severity T1 and T2, whilst also determining whether hyposalivation has occurred in accordance to oral mucositis development.

- To analyse clinical oral mucositis presence and severity during IMRT, 2 weeks after, 6 weeks after, 12 weeks after IMRT.
- All of the above were related longitudinally to the baseline pre-IMRT scores where applicable (T0) – the patients' acted as their own controls.

2.3 Materials and Methods

2.3.1 Ethical Approval

This study was approved by the North of Scotland Research Ethics Service (NRES) Committee foundation in October 2016 (16/NS/0116), the Health Research Authority NHS (IRAS Project ID:199100) on 21st January 2017 and finally, the confirmation of capacity and capability to conduct research at Guy's and St Thomas' NHS Foundation Trust (GSTFT) was granted in February 2017.

All participants enrolled in the study gave informed consent and signed appropriate documentation to this effect.

All volunteer samples for this study were obtained from the Special Care Dental Unit at Guy's Dental Hospital, London, UK.

2.3.2 Participants

Head and Neck Cancer (HNC) Patients pre-IMRT

Head and neck cancer (HNC) patients over the age of 18, with permanent dentition, both genders, at any stage of disease, except where distant metastases were present requiring high-dose RT treatment of the primary tumour, without systemic diseases affecting the salivary flow and oral host defence mechanisms (Table 1).

2.3.2.1 Exclusion Criteria

Volunteers younger than 18 years old (non-permanent dentition), unable to give consent and volunteers who were administered antibiotics in the previous 3 months (affecting oral microbial composition, altering and promoting the opportunistic colonization)(Willing et al., 2011) were excluded. Volunteers with uncontrolled systemic disease were also excluded from this study (Carpenter et al., 2000; Gil-Montoya et al., 2008; Izumi et al., 2015; Gil-Montoya et al., 2016).

Table 2. 1 Inclusion and exclusion criteria.

Inclusion Criteria	Exclusion Criteria
HNC Patients: Male - Female Over the age of 18 Received radiation treatment for HNC	Younger than 18 Unable to give consent Administered antibiotics in the previous month Patients with non-controlled systemic disease Patients with Sjogren syndrome. Patients with Rheumatoid arthritis Neurological disorders (Parkinson's disease) Dehydration Sialoadenitis Sialolithiasis

2.3.2.2 Sample Size Calculation

The power calculation for this study was based on comparing all the clinical and biochemical parameters analysed in HNC patients before and after treatment. Previous studies investigated salivary biochemical composition without a sample size calculation, therefore no previous data were available to support power calculations (Funegård et al., 1994; Almståhl et al., 2001; Eliasson et al., 2005; Hannig et al., 2006; Vidotto et al., 2010; Dijkema et al., 2012; Laheij et al., 2015; Richard et al., 2017).

Statistical advice was obtained prior to start this study in order to determine the sample size required by a suitable power calculation. The power calculation was based on comparing the mean protein levels in head and neck cancer patients before and after IMRT. A study with

80% power at 5% level of significance (Chaudhury et al., 2015) will be able to detect the true difference in all parameters before and after cancer treatment with an effect size of 0.5 (high according to Cohen 1988) using two tailed test would require a total sample of 35 patients. The power calculation was carried out using Gpower 3.1.5. software (Franz Faul, Universitat Kiel) (Faul et al., 2007)

2.3.2.3 Participant Recruitment

Patients diagnosed with HNC at Guy's and St Thomas' NHS Trust were recruited and gave informed consent prior to sample collection. In the longitudinal study all the samples were collected at three time points. Firstly, 40 HNC patients were recruited before their cancer treatment started (pre-IMRT, T0), 38 patients were examined at 6 months after IMRT (± 1 month, T1), and 33 patients at one-year post-IMRT (T2).

2.3.2.4 Demographics and Clinical Characteristics

Demographics and clinical characteristics of the volunteers participated in this study:

Age, gender, smoking and drinking habits were reported by patients.

Type of treatment received, total dose, fractionation of radiotherapy.

Medical history, it was used patient records as a source of information about the medical experiences and current medication intake.

2.3.2.5 Tumour Localization and Stage of Cancer

Tumour localization and the stage of cancer prognosis is related to severity and extension of the disease. The staging assessment of HNC uses the rules on classifications that were publish by the American Joint Committee on Cancer (AJCC) which is the Tumour Node Metastasis (TNM) classification. TNM is based on three anatomical components, the extent of the

primary (T), describes the evidence of lymph nodes involvement or (N) and the possibility of cancer spreading to distant part of the body (M)(Chi et al., 2015; Roland et al., 2016).

Tumor histology was recorded from the clinical history reported by the oncology team.

2.3.3 Sample Collection

The following samples were collected:

- unstimulated whole mouth saliva (UWMS), saline solution rinse
- plaque biofilm
- mucosal swabs collecting residual saliva from the inner cheek
- excavated carious dentine tissue and/or extracted carious teeth.

Sample collection added 15 min to the conventional treatment time. Samples were collected during the day, routinely between the hours 1:30pm to 3:30pm in the dental clinic in an attempt to avoid diurnal variations.

2.3.3.1 Unstimulated Whole Mouth Saliva Sample Collection (UWMS)

Unstimulated salivary flow rate reports the basal secretion of salivary glands without external stimuli (Proctor 2016). All participants had refrained from food and drink for at least 30-60 min prior to sample collection. UWMS samples were obtained by passive drooling (Navazesh,1993) into a pre -weighed 50 mL sterile conical polypropylene tube (Falcon; BD Biosciences, San Jose, CA, USA) for 10 minutes.

All subjects were seated in a dental chair and they were instructed to swallow, prior to leaning their head forward and allow saliva to collect in the mouth, and let the saliva start to drain into the tube. All sample collections were carried out at the same time of the day and under similar clinical conditions in order to exclude circadian variations in salivary flow rate and composition (Dawes and Ong, 1973). The samples were placed immediately on ice, keeping the temperature at 4°C, to avoid proteolysis and deglycosylation of the salivary proteins. After collection, samples were transferred to the laboratory where the tubes were re-weighed and

the net saliva volume was calculated. Saliva samples were aliquoted into microtubes 2ml (Mikro-schraubrohre 2ml, PP. Sarsted AG & Co, KG, Numbrecht Germany), labelled and stored in – 80 °C freezers for further analysis.

An alternate saliva sample was collected from each participant, in order to combat hyposalivation, a possible side effect that HNC patients may develop after radiotherapy. Each participant was instructed to rinse 0.9% normal saline solution (5 ml) for 30s and then spit into a 50 mL sterile conical polypropylene tube (Falcon; BD Biosciences, San Jose, CA, USA).

The samples were placed immediately on ice, keeping the temperature at 4°C, to avoid proteolysis and deglycosylation of the salivary proteins. After collection, samples were transferred to the laboratory labelled and stored in – 80 °C freezers for further analysis.

2.3.3.2 Unstimulated Whole Mouth Saliva Flow Rate

Flow rate was measured by reweighing the sample tubes and subtracting it from the pre-weighed tubes to get the net saliva weight in grams, saliva density of 1.0 g/ml.(Ligtenberg et al., 2015). The net weight was divided by the duration of sample collection (10minutes) to measure salivary volume secreted per minute (ml/min).

2.3.4. Clinical Oral Assessment

An oral clinical assessment was performed on all participants.

2.3.4.1 Dry Mouth and Taste Report

Dry mouth feeling was established based on the patients' answers of the following questions extracted from the questionnaire validated by Fox et al (Fox et al., 1987):

- Does the amount of saliva in your mouth seem to be less than normal? yes/no

  This question was asked at every time point post IMRT in order to determine prevalence of this symptom longitudinally after one year of treatment. Furthermore, to assess the possible association with salivary flow rate, dental assessment, taste perception, oral mucositis and in the following chapter with salivary protein composition.

Taste variation was established based on patients' reports, answering the following question extracted from the Late Effects of Normal Tissue/Somatic Objective Management Analytic (LENT/SOMA) scoring system:

- Has your sense of taste changed after IMRT? Can you taste the food in the same way as before radiotherapy? yes/no

  This taste perception was measured at every time point post IMRT to assess its prevalence and association with the other oral/dental assessment results.

2.3.4.2 Oral Mucositis Grading

The most common assessment scales used are The National Cancer Institute (NCI)-Common Toxicity Criteria (CTC version 4.0), The European Organization for Research and Treatment of Cancer (EORTC), The Toxicity criteria of the Radiation Therapy Oncology Group (RTOG) the criteria developed by World Health Organization (WHO) in 1979among others (Sonis, 2004b; De Sanctis et al., 2016; Villa and Sonis, 2016).

The World Health Organization (WHO) Oral Toxicity Scale, for example, combines signs of mucosal damages (erythema and ulceration) with functional impairment, while the RTOG criteria are based only on a general description of mucosal damage intensity.

In the study clinical assessment of oral mucositis was performed by the oncology team during and after the radiotherapy adding three time points post IMRT (2 weeks, 6 weeks and 3 months) using the World Health Organization (WHO) oral toxicity scale (World Health Organization, Handbook, 1979). This grading system is based on a clinical examination of the oral cavity combining signs of erythema and presence of ulcers with questioning the patient about pain and their diet to assess functionality.

The scoring scale is grade 0: no oral mucositis; grade 1: erythema and soreness (no ulcer); grade 2: ulcer (s) present, subject able to eat solids; grade 3: ulcer (s) present, subject requires a liquid diet (due to mucositis); and grade 4: ulcer (s) present, alimentation not possible (due to mucositis) (Figure 2.1 ;WHO organization 1979 handbook).

The WHO scale presents a complete assessment of the range of signs and symptoms of mucositis, but it depends on subjective physicians' judgement and evaluation (De Sanctis et al., 2016; Villa and Sonis, 2016).

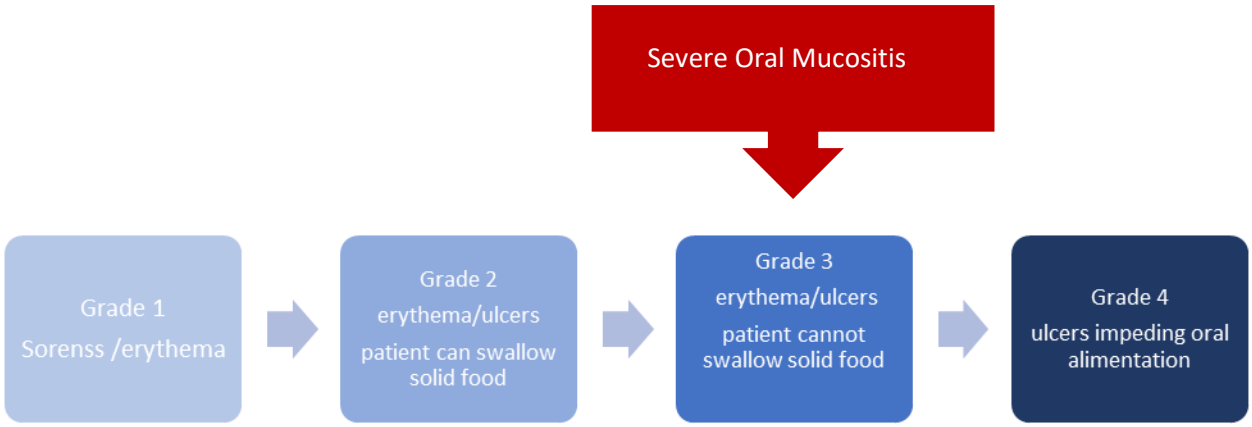


Figure 2. 1 The WHO Oral Toxicity Scale.

Another important factor to take in account to choose among grading systems is which scale does not fatigue or increase patient pain.

2.3.4.3 Dental Assessment

The number of carious lesions present from clinically visible change of colour to dark brown on any surface to open lesions in the oral cavity. Number of teeth / decayed, missed or filled (DMFT) were included in the clinical assessment. This clinical and visual index has been used for more than 80 years as a diagnostic criterion in epidemiology, in order to detect and quantify the total number of teeth affected by carious lesion (Fejerskov, 2004; Kidd and Fejerskov, 2004). This method relays on clinical experience of the examiner, however it is rapid, economical and used around the world. In addition, it is satisfactory for detecting enamel lesions on accessible surfaces, dentinal lesions (non cavitated) and cavitated dentinal caries(Pitts, 2001; Pitts and Stamm, 2004).

At each time point participating patients were clinically examined by the same consultant in the Special Care Dentistry Unit, at Guy's and St Thomas' Hospitals NHS Foundation Trust. Special Care Dentistry is the specialist unit for care of head and neck cancer patients. The consultant examining the patients is the clinical lead for dental oncology service and Sedation as well as Special Care Dentistry. Additionally, this clinician has been part of the guidelines' development group for the oral management of oncology patients. The consultant performing the dental assessments was calibrated on caries detection (DMFT) in 2011 using the Adult Dental Health Survey criteria (O'Sullivan 2011).

Visual examinations of the participating head and neck cancer patients were performed before cancer treatment (T0) and post IMRT at two time points (T1 and T2). Panoramic radiographs were done before start of radiotherapy, but at the end of the cancer treatment radiographs were taken only when needed to assist patient care and management decision-making.

Following the recommendations regarding dental care for HNC patients undergoing IMRT at Guy's and Thomas a dental assessment before starting the treatment is mandatory. This is a baseline visit, with the multidisciplinary dental team pre IMRT, mandatory for all patients in

order to plan and deliver a care pathway. The assessment includes a dental exam to identify any oral diseases and remove infections, hygienist support and preparation for side effects of cancer therapy. At special dental care unit all patients will receive a prescription of a sodium fluoride toothpaste (Duraphat® 5000) for twice daily use. Six months after starting cancer therapy patients had a dental appointment in order to assess oral function, side effects and risk factors along with a reinforcement of oral hygiene and use of fluoride toothpaste. After this second appointment patients were usually discharge to primary practitioner care. However, in this study patients were seen 12 months after the start of IMRT adding one follow up to the regular care.

Clinical diagnosis criteria of carious lesion were performed by the same calibrated consultant at every time point following this protocol, it was defined as a sound tooth / surface, those that had no visible caries after clean and dry teeth surfaces. Regarding teeth affected by carious lesion the threshold were defined at it follows, D1 indicating incipient enamel lesion with an intact surface, D2 representing cavitated enamel lesion and D3 as a clinically detectable dentine lesion presenting cavitated enamel and dentin exposure (Pitts, 2001; Pitts and Stamm, 2004; Pitts et al., 2017; Sroussi et al., 2017). Lesion were recorded at these 3 stages.

In addition, it was reported the number of surfaces affected by caries as it follows, it was analysed a total number of five tooth surfaces on the posterior teeth and four tooth surfaces on the anterior teeth, determining the total number of sound and carious surfaces at every time point through clinical visual observation (Sroussi et al., 2017).

It is important to remark that post radiation carious lesions affect nonclassical surfaces of teeth such as cusp tips, occlusal and incisal edge, cervical line and buccal and oral smooth surfaces.

Radiotherapy caries clinical appearance starts as brown stain decolouration spot (incipient uncavitated lesion) after a rapid onset caries development and progression present a high destructive potential leading to amputation of crowns in a short period of time resulting in to edentulousness and therefore a reduced oral function and quality of life (Jansma et al., 1989; Silva et al., 2009; Lieshout and Bots, 2014).

One of the limitations of this scoring system is that depends on clinician knowledge and experience which could add variability among the examiners. This method of detecting caries is only a report whether caries is present or not, it does not provide any information regarding activity or carious lesion stage(Pitts, 2001; Pitts and Stamm, 2004).

Additionally, when using this index, the missing tooth and filled components are still under debate, due to it does not calculate the number of teeth lost or filled for reasons other than carious lesion. As a result, it can overestimate caries experience in a specific group (Broadbent and Thomson, 2005).

2.3.5 Statistical Analysis

Descriptive statistics was used to summarise the sample characteristics and other clinical factors. The clinical parameters, protein concentration and secretion rate found in HNC patients before and after treatment were tested using:

- Repeated measures Friedman test to assess variation of clinical parameters, salivary flow rate, total protein concentration, secretion rate and specific proteins in saliva within the oral cavity of HNC patients at three time point.
- Data in different categories was analysed using Wilcoxon matched pairs test for testing the differences between same patient.
- Data in different categories was analysed using Mann-Whitney test to compare differences between two independent groups.
- Data of different tumour location was analysed using Kruskal-Wallis test to compare differences between independent groups.
- Descriptive statistics were used to present baseline demographics, dental assessment, patient-reported xerostomia, taste changes and oral mucositis.
- Random effects Generalized Least Squares linear regression.

Random effects linear regression analysis in a longitudinal panel is a model which was used to analyse the data obtained from several measures taken from the same patients at different

times, in order to determine the relationship between two variables making a distinction between independent and dependent variables.

Panel data refers to a group of patients that were studied recurrently over time in order to determinate the association between salivary flow rate, dry mouth reports taste changes reports and oral mucositis development at T1 (6 months post IMRT) and T2 (12 months post IMRT) in comparison to T0 (pre IMRT).

All analysis was carried out using STATA 15.1 (College Station, Texas USA, www.stata.com), GraphPad Prism 8 software (La Jolla California USA, www.graphpad.com) and Microsoft Excel 2018, after checking for normality. Normality of the data was tested for all the parametric analyses using histogram and box plots. Results were expressed as a mean ± SEM relative to the variables and 'n' represents the number of subjects, $p < 0.05$ value was considered statistically significant.

2.4 Results

2.4.1 Patient Demographics

Demographics and clinical characteristics of the HNC patients is *shown is Table 2.2*

Table 2. 2 Patient Demographics. Data is expressed as mean ± S.E.M.

Patients Recruited	40
Age (Years)	62.562 (± 2.15)
Range	44 - 75
Gender	
Male	36
Female	4
Habits	
Smoking	Yes 28 No 8 Unknow 6
Drinking	Yes 22 No 9 Unknown 9

Table 2.3 Tumour Histology

Primary Tumour histology	Number of patients
Squamous cell carcinoma	38
Adenocarcinoma	1
Unknown	1

Table 2.4 Treatment

Treatment	Patients	Mean dose	30 fractions	20 fractions	12 fractions
IMRT	40	62.38	36	3	1
Chemotherapy	19	2			
Cisplatin	18				
Carboplatin	1				

2.4.2 Tumour Characteristics

The tumour localisation and the stage of cancer prognosis was evaluated using the TNM classification (Table 2.5 and Table 2.6).

Table 2.5 Primary Tumour Location.

Tumour Location	Number of Patients
Tonsil	12
Oropharynx	6
Tongue	5
Nasopharynx	2
Hypopharynx	3
Larynx	3
Neck	2
Epiglottis	2
Parotid	1
Nasal	1
Mandibular	1
Buccal mucosa	1
Sinus	1

Table 2.6 Tumour staging

Tumour Staging								
T	TX	T0	T1	T2	T3	T4		Unknown
	0	1	3	**15**	4	13		4
N	N0	N1	N2	N2a	N2b	N2c	N3	
	10	3	3	2	**13**	4	1	4
M	M0							
	36							4

Table 2.7 Medication

Medication	Number of patients
Not reported	7
Bisphosphonates	4
Immunosuppressive agent	6
Steroid	9
High blood pressure (β- blocker)	13
No medication	5

2.4.3 Dropout Rate

In general, patient compliance was positive with the majority of the patients receiving their scheduled dosages as originally planned.

40 HNC patients were recruited before radiotherapy started. In total, 4 patients died during the study, one patient died before T1, and 3 more before T2.

The total drop-out figure was 7 participants, including one due to antibiotic usage and 2 who did not comply to their scheduled appointments.

2.4.4 Unstimulated Whole Mouth Saliva (UWMS) Flow Rate (FR)

Mean salivary flow rate (SFR) of HNC patients prior to IMRT (T0) was 0.44 ± 0.04 ml/min, similar to the resting flow rate of healthy patients (p = 0.1253) (Figure 2.2).

At 6 months post-IMRT (T1), the salivary flow rate was statistically significantly decreased to 0.16 ±0.02 ml/min, (p = 0.0001) compared with pre -radiotherapy salivary flow rate (Figure2.2.).

At 12 months post-IMRT (T2), the salivary flow rate was increased in comparison to T1, reaching 0.24 ±0.03 ml/min. The mean SFR values showed at T2 represents 52.79% of the original values of T0 (Figure 2.2).

Only 6 subjects showed a full recovery of salivary flow rate values compared with T0 mean values, whilst 20 subjects recovered less than 50% of the salivary flow rate pre-IMRT mean values.

Analysis of salivary flow rate post IMRT among different locations showed no statistical differences at T1 (p= 0.5564) and T2(p=0.6125) (Kruskal-Wallis test)

Analysis of salivary flow rate post IMRT among different medication showed no statistical differences at T0 (p= 0.443) T1 (p= 0.452) and T2(p=0.676) (Kruskal-Wallis test)

Analysis of salivary flow rate after IMRT showed a significant reduction compared with baseline (p <0.0001) (Friedman Test).

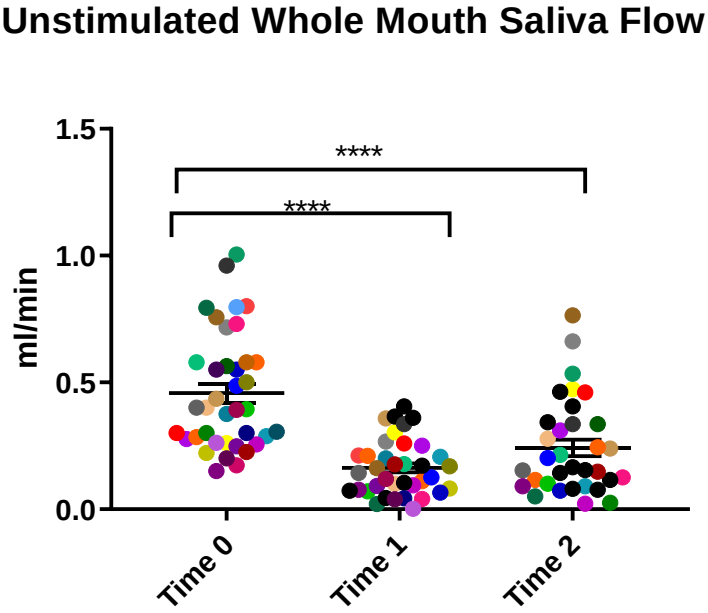


Figure 2. 2 Salivary flow rate variation pre- (T0) and post-IMRT at 6 months (T1) and 12 months (T2).

UWMS flow rate was reduced significantly at T1, compared with T0 ($p<0.0001$) (Wilcoxon matched pairs signed rank test). UWMS flow rate was decreased significantly at T2 compared with T0 (p0.0018). Every colour represents 1 HNC participant. Data is represented as mean ± SEM.

2.4.5 Xerostomia Perception

Dry mouth feeling was assessed based on the HNC patients' answers of the Fox et al questionnaire (Fox et al., 1987), this questionnaire has been used by in order to complement the information obtained by measuring salivary flow rate, which is essential in salivary hypofunction diagnosis. Additionally, determine the association between this subjective condition reported by patients and the objective measurement of salivary quantity and quality.

Therefore, among several questionnaires fox et al (1987) developed this survey to predict hyposalivation (Villa et al., 2014), this particular question was selected due to the simplicity to identify patients with symptoms of xerostomia regarding the amount of saliva by answering yes or no, in order to report an overall feeling of oral dryness. The whole questionnaire would add extra time to the clinical appointment, it would be needed a second examiner capable of collecting that information probably it would be necessary an extra session which in this case is not suitable for the patient's urgency of being treated and the multiple medical controls that they have to attend in a short period of time.

Dry mouth feeling was established based on the patients' answers of the following question extracted from the questionnaire validated by Fox et al(Fox et al., 1987):

- Does the amount of saliva in your mouth seem to be less than normal? yes/no

As a result, 92% of patients (n= 35) reported dry mouth and 8% of patients (n=3) reported no sensations of dry mouth post-IMRT at 6 months (T1). This is in comparison to pre-IMRT (T0) where no patient reported a dry mouth sensation 94% of patients (n= 31) reported dry mouth sensation and 6% patients (n=2) reported no sensations of dry mouth, at 12 months post-IMRT (T2) This is in comparison to T0 where no participants at all reported dry mouth sensations.

There is no statistically significant difference between perceptions of dry mouth between T1 and T2.

2.4.5.1 Longitudinal Associations Between Salivary Flow Rate and Dry Mouth Feeling Reported by Patients Post IMRT at 6months (T1) and 12 Months (T2) by using Random effects Generalised Least Squares linear regression

Analysis of associations between UWMS flow rate and dry mouth reported by patients pre- and post-IMRT (Table2.8) shows a negative and significant association between dry mouth reported by patients and salivary flow rate post-IMRT at T1, but no significance at T2.

Table 2.8 Association between salivary flow rate (SFR) and dry mouth reported by patients post-IMRT at 6 months (T1) and 12 months (T2).

Dry Mouth Feeling/SFR	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	-0.0973	0.220	-0.252	0.0580
T1	-0.202	**0.011**	-0.359	-0.046
T2	-0.121	0.135	-0.281	0.038

Table shows significant association between dry mouth feeling reported by and salivary flow rate post-IMRT at 6 months(T1) (p=0.011). No significant association at 12 months (T2) and (p=0.135).

2.4.6 Hypogeusia / Dysgeusia

Changes in taste perception reported by patients by answering one question extracted from the Late Effects of Normal Tissues (LENT)–Subjective, Objective, Management, and Analytic (SOMA) system (LENT/SOMA) scoring system which is a validated scoring system used by National Cancer Institute's (NCI) in the UK to assess treatment side effect in cancer patients. this is a frame of reference for assessment and grading of IMRT side effects.

There is a specific one for HNC patients it has three different parts that includes demographic assessment type of cancer and treatment, the second part is a subjective oral assessment the following question was extracted from that section, which included 38 different questions. The last part of this questionnaire is including an evaluation of cancer treatment side effects including skin, mucosa and teeth (50 questions).

Variation in taste perception was established based on the patients' answers of the following question extracted from the questionnaire validated system LENT/SOMA

- Has your sense of taste changed after IMRT? Can you taste the food in the same way as before radiotherapy? yes/no

35% of HNC patients (n=13) reported maintained sensations of taste, at T1, whereas 65% of patients (n=24) reportedly losing their sense of taste at T1. For comparative purposes, prior-IMRT (T0) all patients reported ordinary levels of taste. No statistically significant difference regarding taste between T1 (6 months post IMRT), & T2 (12 months post IMRT), was reported by HNC patients. 52% of HNC patients (n=17) reported maintained sensations of taste, at T2, whereas 48% of patients (n=16) had lost their sense of taste.

2.4.6.1 Longitudinal Associations Between Taste Reported by Patients and Salivary Flow Rate Post IMRT at 6months (T1) and 12 Months (T2) by using Random effects Generalised Least Squares linear regression

Presence of taste alteration (dysgeusia) reported by patients and UWMS flow rate at T1 and T2, revealed a significant association between taste variation reported by patients and salivary flow rate post-IMRT at T1 and T2 (Table 2.9).

Table 2.9 Presence of taste alteration (dysgeusia) reported by patients and Salivary flow rate in UWMS post IMRT at 6 months (T1) and 12 months (T 2).

Taste Change Report/SFR	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	0.237	0.274	-0.188	0.662
T1	-0.570	**<0.0001**	-0.771	-0.368
T2	-0.398	**<0.0001**	-0.584	-0.212

Table shows a significant relation between taste variation reported by patients and UWMS flow rate in after IMRT at T1 and T2 compared with T0 (pre-IMRT).

2.4.7 Oral Mucositis

Oral mucositis evaluation was performed by the clinical oncology team at different time points using WHO oral toxicity scale to quantify oral mucositis presence and severity based on clinical signs and symptoms and type of food intake which indicated a functional measure.

Oral assessment at special dental care was performed pre IMRT (T0) at 6 months (T1) and one-year post IMRT (T2). Clinical examinations were undertaken by the same consultant (specialist dentist) who carried out the dental exam at Sedation and Special Care Dentistry Unit at Guy's and St Thomas' Hospitals NHS Foundation Trust.

In addition, patients recruited were assessed during the IMRT period, 2 weeks, 6 weeks, 3 months, 6 months and one-year post IMRT by the oncology team at Guy's Hospital using the WHO index to determinate presence and severity of oral mucositis**.**

Prior-IMRT (T0) at T0 there was no patients with oral mucositis, during RT 31 patients presented oral mucositis, two weeks after the treatment was finished 27 patients had mucositis. At six weeks post IMRT 15 patients still presented the condition. Finally, six months post IMRT, (T1), the vast majority of participants did not have oral mucositis (n= 32) and 16% participants (n=6) had oral mucositis, at T1. At T2, 91% of participants (n= 30) did not have oral mucositis and only 9% participants (n=3) had oral mucositis, 12 months post IMRT (T2) (Figure 2.3). There was no statistically significant difference between T1 & T2 (p= 0.625) (Wilcoxon matched pairs signed rank test) Percentages were rounded to nearest whole number percentage.

Among the 31 patients that developed oral mucositis during IMRT, sixteen subjects reached grade 2 and thirteen grade 3 during and after IMRT as a maximum value (Table 2.10), at T1 (6 months post IMRT) six patients developed grade 1 and at T2 (12 months post IMRT) three patients developed grade 1, showing a significant drop in number of patients that presented oral mucositis (p=<0.0001) in comparison to the number of patients that presented that side effect during and at the early time-points (2 weeks) after IMRT. According to WHO mucositis scale grade 1 mucositis is considered as a mild side effect without presenting any ulcer that can cause pain (Maria et al., 2017).

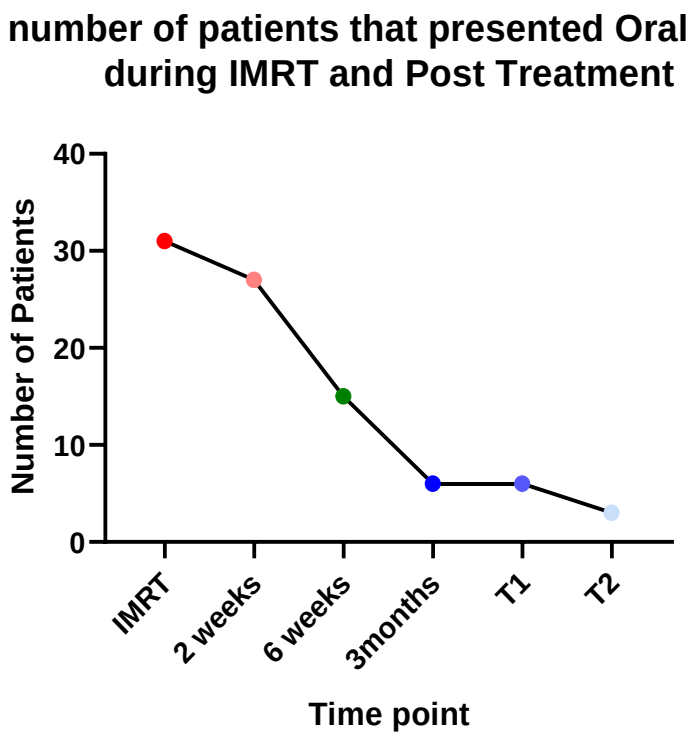


Figure 2.3 Number of patients that present oral mucositis at different time points.

From 6 weeks post IMRT the number of subjects presenting this condition is significantly reduced in comparison with the numbers observed during IMRT ($p<0.0001$). However, there was no significant difference 2 weeks post IMRT compared with during IMRT ($p= 0.1486$) (Mann-Whitney test). Data presented in numbers.

Table 2.10 Oral mucositis assessment during and after IMRT by primary tumour location.

Tumour location	During IMRT	2 weeks	6 weeks	3months	Max.
Left oropharynx	3	2	2	0	3
Bilateral oropharynx	2	2	0	0	2
Right oropharynx	3	2	2	1	3
Oropharynx	2	3	0	0	3
Left oropharynx	2	3	2		2
Left oropharynx					
Left tonsil	3	3	1		3
Right tonsil	2	2	3	0	3
Left tonsil	3	1	0	0	3
Left tonsil	2	2	1	1	2
Right tonsil	1	3	2	1	3
Left tonsil	2	1	0	0	2
Left tonsil	1	2	1	0	2
Right tonsil	2	2	1	1	2
Left tonsil	2	0	1	0	2
Left tonsil	2	1	1		2
Right tonsil	2				2
Right tonsil	1	2	2	0	2
Left hypopharynx	1	1	0	0	1
Pharynx	0	0	0	0	0
Left hypopharynx	3	2	1	1	3
Bilat nasopharynx	3	2	2	2	3
Nasopharynx					
Larynx	0	0	0	0	0
Right larynx	2	0	0	0	2
Larynx	2	2			2
Right neck	1	1	0	0	1
Left neck	3	3	1	0	3
Parotid					
Right maligns sinus	2	2	1	0	2
Nasal and mid ear	3	2			3
Anterior mandibular					
Left buccal mucosa	2	1	0	0	2
Bilateral tongue	2	2	0	0	2
Right tongue	3	3			3
Border tongue					
Base of tongue	2	2	2	0	2
Right tongue	3				3
Epiglottis	3	0	0	0	3
Left epiglottis					

Table shows the different grades of oral mucositis among patients separated by primary tumour location during and post IMRT, and the maximum grade reached at any time point.

In this study there was no significant difference in the radiation dose received for these patients (mean 62.5 Gy), as well as the fractioning planning reporting 30 fractions in 37 patients, the location of the tumour varied reporting mainly tonsil, pharynx (oropharynx, nasopharynx and hypopharynx) and tongue reaching 28 subjects. However, during IMRT there was no statistical difference in mucositis onset and severity regarding primary tumour location (Man-Witney test).

2.4.7.1 Longitudinal Associations Between Salivary Flow Rate and Oral Mucositis Post IMRT at 6 months (T1) and 12 Months (T2) by using Random effects Generalised Least Squares linear regression

The outcome measures of oral mucositis status of HNC patients post-radiotherapy along with a reduced volume of saliva secretion have been presented in this chapter as well as the relation between these two outcomes at time point 1 and 2. It was showed that the reduced salivary flow rate was negatively associated to oral mucositis development.

Analysis of oral mucositis and UWMS flow rate in at 6 months (T1) and 12 months (T2), revealed a significant association between oral mucositis and salivary flow rate post-IMRT (Table 2.11).

Table 2.11 Presence of oral mucositis and Salivary flow rate in UWMS post-IMRT at 6 months (T1) and 12 months (T2).

Mucositis/Salivary flow rate	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T1	0.001	0.987	-0.125	0.127
T1	-0.287	**0.0001**	-0.354	-0.221
T2	-0.213	**0.0001**	-0.280	-0.146

Table shows a significant association between oral mucositis and salivary flow rate in UWMS after IMRT at T1and T 2.

2.4.7.2 Longitudinal Associations Between dry mouth feeling reported by patients and oral mucositis post IMRT at 6months (T1) and 12 months (T2)

Analysis of dry mouth feeling reported by patients and oral mucositis at 6 months (T1) and 12 months (T2), revealed a significant association post-IMRT (Table 2.12).

Table 2.12 Presence of oral mucositis and dry mouth feeling post-IMRT at 6 months (T1) and 12 months (T2).

Dry mouth feeling/ Mucositis (WHO)	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	0.073	0.343	-0.078	0.226
T1	0.911	**0.0001**	0.827	0.995
T2	0.931	**0.0001**	0.846	1.017

Table shows a positive and significant association between oral mucositis presence and dry mouth feeling reported by patients after IMRT at T1 and T 2.

2.4.8 Dental Assessments

The total number of teeth present was significantly reduced at T1 (6 months post-IMRT) & T2 (12 months post-IMRT), in comparison to T0 (pre-IMRT) (p <0.0001) (Figure 2.4). No significant differences were observed in the quantity of teeth between T1 and T2.

The total number of teeth that were visibly missing, due to extraction, was significantly increased at T1 & T2 in comparison to T0 (p<0.0001) (Friedman test) (Figure 2.5)

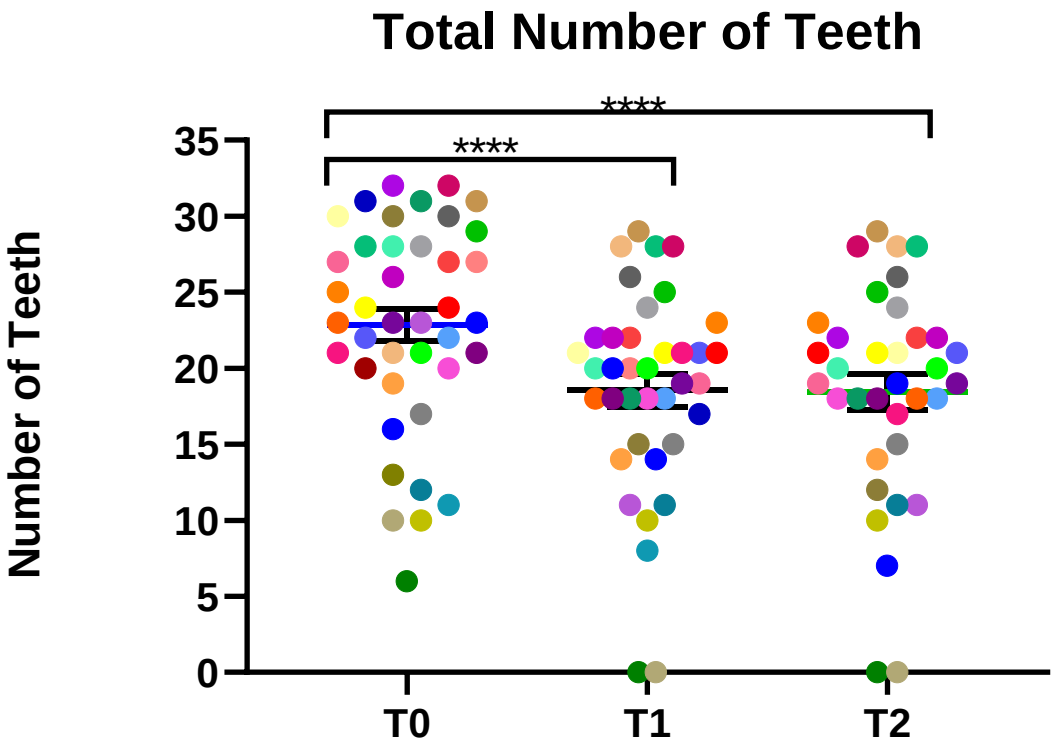


Figure 2.4 Number of teeth present in patients' oral cavities variation pre- (T0) and post-IMRT at 6 months (T1) and 12 months (T2).

The total number of teeth present was significantly reduced at T1 & T2 in comparison to T0 ($p < 0.0001$) (Wilcoxon matched pairs signed rank test). No differences were observed in the quantity of teeth between T1 and T2. Data is represented as mean ±SEM.

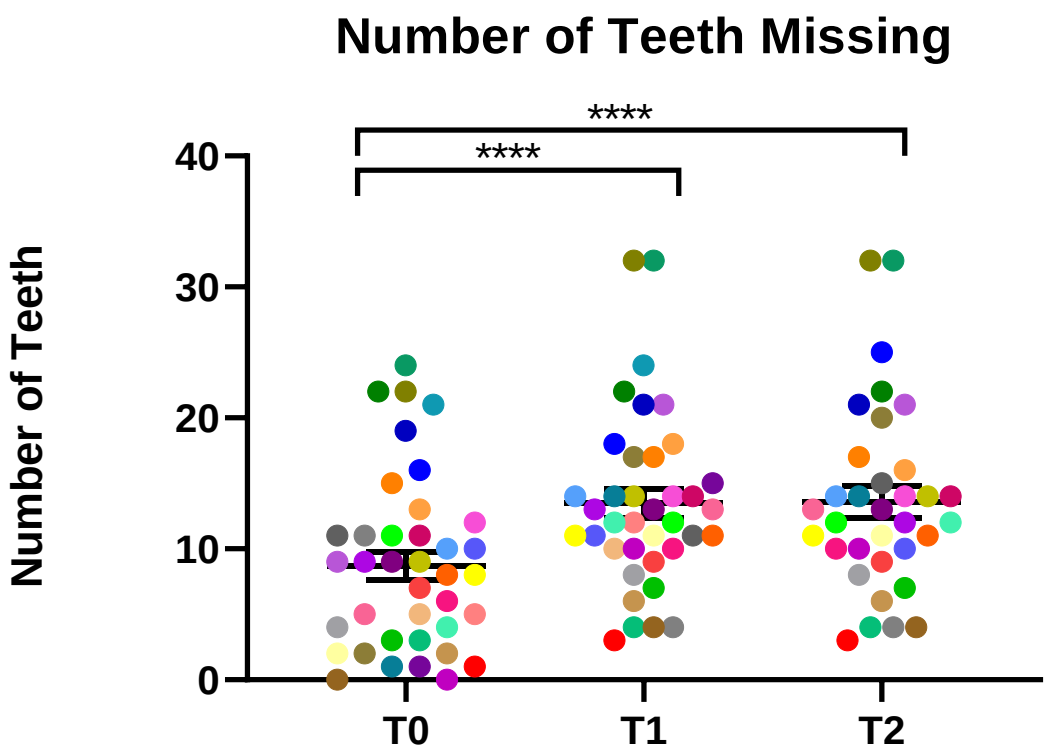


Figure 2.5 Number of Teeth Missing pre- (T0) and post-IMRT at 6 months (T1) and 12 months (T2).

The total number of teeth that were missing, due to extraction, was significantly increased at T1 and T2 in comparison to T0 ($p < 0.0001$, Wilcoxon matched pairs signed rank). No statistically significant differences were observed in the quantity of teeth missing between T1 and T2. Data is represented as mean ± SEM.

Dental assessment revealed the total number of teeth that were without any type of visible restoration and without clinical caries present was significantly decreased at T1 and T2, in comparison with T0 (p=0.0418 and *p*= 0.0014 respectively) (Figure2.6).

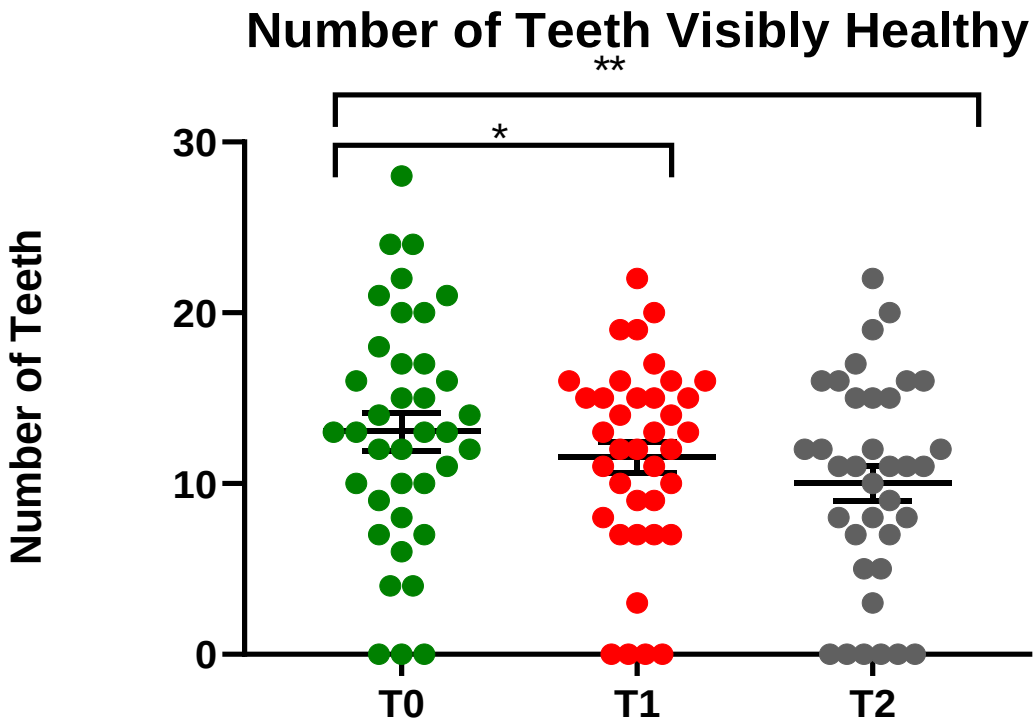


Figure 2.6 Number of teeth visibly healthy at T0, T1 and T2.

The total number of teeth that were without any type of visible restoration and without clinical caries present was significantly decreased at T1 & T2 in comparison to T0 (p = 0.0418 and p= 0.0014) respectively. Wilcoxon matched pairs signed rank test) No statistically significant differences were observed in the quantity of teeth missing between T 1 and T2. Data is represented as mean ± SEM.

The total number of teeth that were visibly affected by development of caries was statistically significantly higher at T0 in comparison to T1 & T2 (p<0.0001 and p = 0.0006). However, at T2 the number of teeth affected by caries lesion was slightly increased (Figure2.7). There is a group of 7 patients at T0 that present 6 or more teeth affected by caries lesion, at T1 this number decrease to two patients reaching 8 and 10 teeth affected, at T2 there were 3 patients and the maximum number of teeth affected were 11. At T0 only 2 patients did not present carious lesions.

The threshold used to determine was from D1 to D3 to asses clinically the presence of carious lesion.

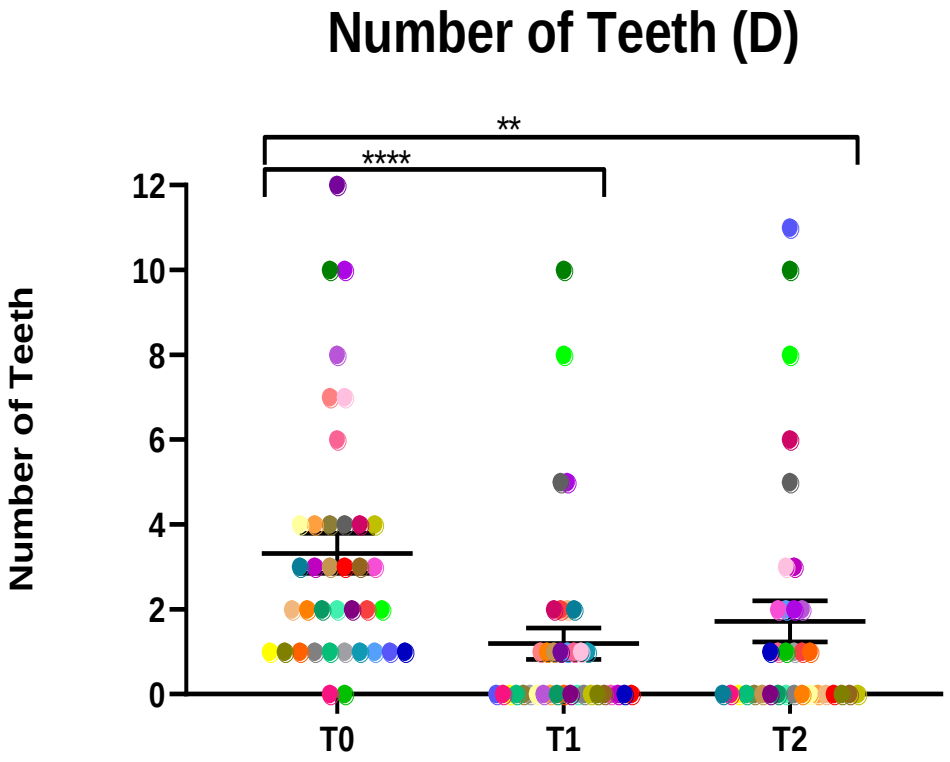


Figure 2.7 Number of teeth with visible signs of caries development pre (T0) and post IMRT at 6 months (T1) and 12 months (T2).

The total number of teeth that were visibly affected by development of caries was statistically significantly higher at T0 in comparison to T1 & T2 (p<0.0001 and p= 0.0) (Wilcoxon matched pairs signed rank test) No statistically significant differences were observed in the quantity of teeth between T1 and T2. Data is represented as mean ± SEM.

Dental assessment pre and post IMRT also revealed the total number of teeth that had filings present was significantly decreased at post IMRT at 6 months (T1) and 12 months (T2) 2 in comparison to pre-IMRT (T0) (p <0.0001) (Friedman Test). No statistically significant differences were observed in the quantity of teeth with restorations between T1 and T2 (Figure 2.8.)

Tooth surfaces that had caries present were significantly reduced at T1 (p <0.0001) & T2 (p<0.0001) in comparison to T0. The same clinical threshold was used to asses number of surfaces D1 to D3. No statistically significant differences were observed in the quantity of surfaces affected by carious lesion at T1 and T2. (Figure 2.9.).

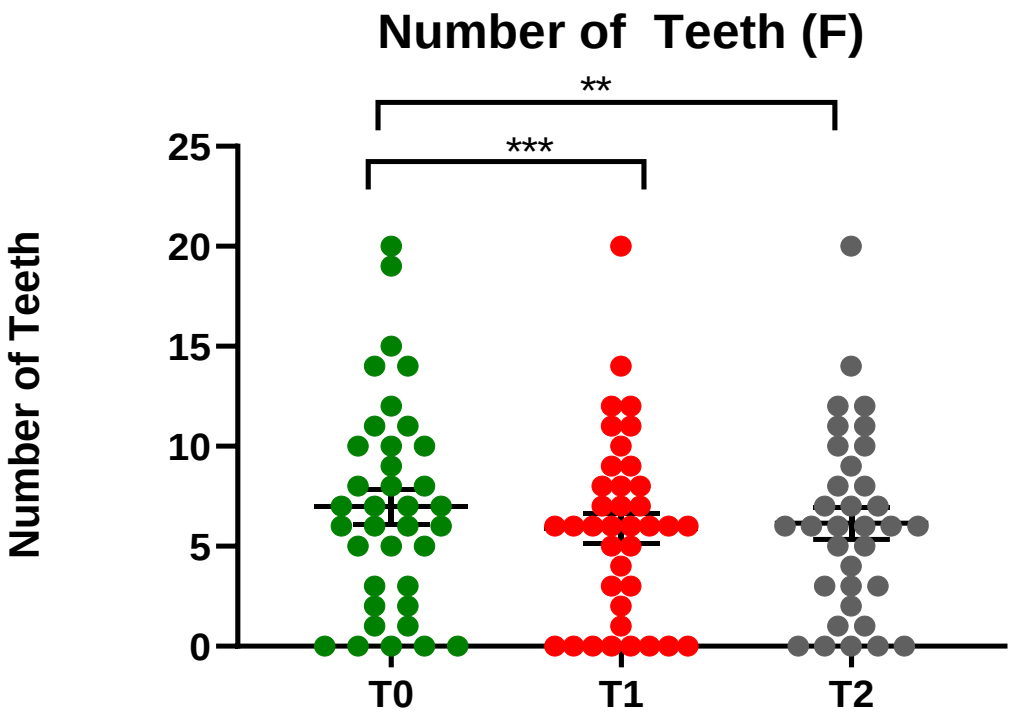


Figure 2.8 Number of teeth with restorations at the three time points measured.

The total number of teeth that had filings present was significantly reduced at T1 & T2 in comparison to T0 (p 0.0007 and p 0.0012). No statistically significant differences were observed in the quantity of teeth with fillings between T1 and T2. Data is represented as mean ± SEM.

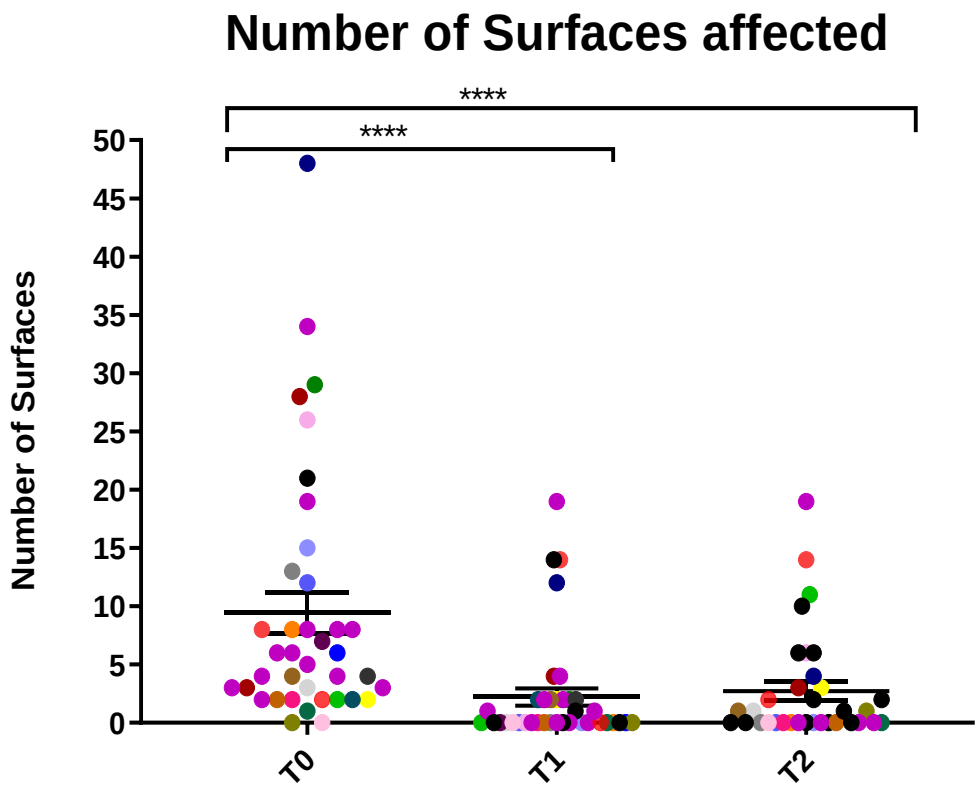


Figure 2.9 Number of tooth surfaces visibly affected by caries at the 3 timepoints measured.

The total number of surfaces on teeth that had caries lesion present was significantly reduced at T1 ($p < 0.0001$) & T2 ($p < 0.0001$) in comparison to T0. (Wilcoxon matched pairs signed rank). No statistically significant differences were observed in the quantity of teeth with fillings between T 1 and T2. Data is represented as mean ± SEM.

2.4.8.1 Longitudinal Associations Between Salivary Flow Rate and Dental Assessment Outcomes Post IMRT at 6 months (T1) and 12 Months (T2) by using Random effects Generalised Least Squares linear regression

Previous studies have reported the dental status related to HNC patients regarding quality of life (Duke et al., 2005). The outcome measures of dental status of HNC patients comparing pre- and post-radiotherapy along with a reduced volume of saliva secretion have been presented. In order to assess the interaction between these two variables, a statistical model that includes the variable of time was used.

The salivary flow rate was significantly reduced post-radiotherapy, UWMS reaching the lowest value at T1 (0.16 ±0.018 ml/min). At T2, showed an increase (0.24 ±0.032) ml/min (Figure.2.2). However, it was significantly lower than T0.

Post-IMRT there was a significant drop in the total number of teeth present in the mouth (Figure 2.4) As well as total number of teeth that were without any type of visible restoration and without clinical caries (Figure 2.6), both showed a strong and negative association with reduced salivary flow rate at T1 and T2 ($p < 0.0001$) (Table2.13 And 2.15).

The number of teeth missing was significantly increased at T1 and T2 (Figure 2.5) representing a negative and significant association with salivary flow rate at both time points ($p<0.0001$) (Table 2.14).

There were strong negative associations between salivary flow rate and the decreased number of teeth that were affected by carious lesions (Figure 2.7) ($p<0.0001$) (Table 2.16) along with the total number of teeth that had restorations present (Figure 2.8.) ($p<0.0001$) (Table 2.17) at T1 and T2. Surfaces on teeth that had carious lesions present (Figure 2.9) were significantly reduced after IMRT and strongly associated to salivary flow rate at T1 and T2 ($p=0.002$ and $p=0.001$ respectively (Table 2.18).

Table 2.13 Number of teeth present in the oral cavity and salivary flow rate post-IMRT at 6 months (T1) and 12 months (T2).

Salivary Flow Rate/ Number of Teeth	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	0.004	0.102	-0.009	0.010
T1	-0.270	**0.0001**	-0.339	-0.200
T2	-0.191	**0.0001**	-0.263	-0.129

Table shows significant association between number of teeth present and salivary flow rate post-IMRT at 6 months (T1) and 12 months (T2) ($p<0.0001$).

Table 2.14 Salivary flow rate and number of teeth missing in the oral cavity post-IMRT at 6 months (T1) and 12 months (T2).

Salivary Flow Rate/ Number of Teeth Missed	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	-0.005	0.079	-0.011	0.00062
T1	-0.267	**0.0001**	-0.337	-0.197
T2	-0.188	**0.0001**	-0.2609	-0.1165

Table shows significant association between salivary flow rate and number of teeth missing post-IMRT at 6 months (T1) and 12 months (T2) ($p<0.0001$).

Table 2.15 Salivary flow rate and number of teeth without clinical caries or restorations present in the oral cavity post-IMRT at 6 months (T1) and 12 months (T2).

Salivary Flow Rate/ Healthy Teeth	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	0.005	0.102	-0.0010	0.0110
T1	-0.285	**0.0001**	-0.349	-0.220
T2	-0.202	**0.0001**	-0.270	-0.1340

Table shows significant association between salivary flow rate and number of teeth without caries and restorations observed clinically post-IMRT at 6 months (T1) and 12 months (2) ($p<0.0001$).

Table 2.16 Number of teeth that present with carious lesions in the oral cavity and salivary flow rate concentration post-IMRT at 6 months (T1) and 12 months (T2).

Salivary Flow Rate/ Number of Teeth (D)	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	5.207	0.194	-2.644	13.060
T1	-5.627	**0.002**	-9.163	-2.091
T2	-5.330	**0.001**	-8.605	-2.055

Table shows significant association between number of teeth affected with carious lesions and salivary flow rate post-IMRT at 6 months (T1) and 12 months (T2) ($p=0.002$ and $p=0.001$ respectively).

Table 2.17 Salivary flow rate (SFR) and number of teeth with restorations in the oral cavity and post-IMRT at 6 months (T1) and 12 months (T2).

Salivary Flow Rate/ Number of Teeth (F)	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	-0.0015	0.736	-0.0104	0.0073
T1	-0.296	**0.0001**	-0.3619	-0.230
T2	-0.0215	**0.0001**	-0.2844	-0.146

Table shows significant association between salivary flow rate and number of teeth with fillings post-IIMRT at 6 months (T1) and 12 months (T2) ($p< 0.0001$).

Table 2. 18 Salivary flow rate (SFR) and number of surfaces with clinical caries present in the oral cavity post-IMRT at 6 months (T1) and 12 months (T2).

Salivary Flow Rate /Number of Surfaces Caries	**Coeficient**	**P**	**[95% Conf.**	**Interval]**
T0	0.0030	0.194	-0.00015	0.007
T1	-0.270	**0.0001**	-0.342	-0.199
T2	-0.192	**0.0001**	-0.265	-0.119

Table shows significant association between salivary flow rate and number of surfaces with clinical caries post-IMRT at 6 months (T1) and 12 months (T2) ($p<0.0001$).

2.5 Discussion

Tumour localisation and staging are two important factors regarding the IMRT beam pattern and final dose received by head and neck cancer patients. RT dose, fractioning and volume of tissue targeted are associated with the risk of developing oral and dental side effects (Villa and Sonis, 2016). In addition, there are patient-related risks, such as past and current dental care, salivary flow rate and composition, oral hygiene, smoking, diet and drinking; that might influence the onset and development of side effects (Sonis, 2013). Therefore, It is important to determine the degree of salivary flow reduction, xerostomia, taste variation and oral mucositis prevalence and severity, following IMRT in order to evaluate these matters further, potentially by even associating these parameters with the IMRT dose and fractioning regiment received by this study's volunteers, along with the possible influence of the different first tumour sites.

In this particular study, the majority of participants received IMRT with no difference in the fractioning regime, with only 4 patients receive a different fractioning regime than the rest of the group. Furthermore, all participants received a mean dose of 62.9 Gy with non-statistically differences among each participant's dosage. Another important factor in this study was the use of patients with varying tumour locations and IMRT treatment sites, and in this case the majority of patients had sites that were in close proximity to each other and anatomically close to parotid and submandibular glands. Therefore, it is possible that these two salivary glands were affected by the toxic side effects of IMRT, with submandibular glands being the main contributor of UWMS (Pedersen et al., 2018).

There were no differences in salivary flow rate among the different locations at T1 and T2 (p (p=0.5564 and p= 0.6125). This is line with previous studies that reported that different IMRT treatment sites had no effect on consequential side effects, and served as a demonstration of how different primary tumour sites are unlikely to affect the xerostomia prevalence when the radiation-dose parameters are matched between the experimental groups (Richards TM (2014). As shown in Figure 2.2, there were statistically significant reductions at both T1 and T2, in comparison T0. Whilst these findings are in accordance with previous studies that utilised conventional radiotherapy, as well as studies of IMRT (Pow et al., 2006; Dijkema et al., 2008, 2010; SHAO et al., 2011; Nguyen et al., 2018), it had been predicted by other studies

that IMRT would produce improved results compared to conventional radiotherapy after one year (Nutting et al., 2011; Nguyen et al., 2018).

One reason for the estimates that IMRT would demonstrate improvements in comparison to conventional radiotherapy, is that past studies (in particular those that demonstrated improvements in flow rate) utilised stimulated parotid gland saliva, or stimulated whole mouth saliva – which contains mainly parotid secretions, which could suggest that parotid glands were spared by IMRT but this data does not denote whether other glands were affected by collateral damage.

In this regard, it is of particular importance to utilise unstimulated whole mouth saliva for measurements of salivary flow rate, as unstimulated whole mouth saliva is a blend of all salivary gland secretions, including the major and minor glands and therefore can offer a more well-rounded picture of the oral environment. Whilst UWMS is produced constantly during the day and night, without external influences, in contrast, stimulated saliva is produced a few times during the day, targeted by external stimuli. Therefore, unstimulated whole mouth saliva is considered more representative of the daily oral situation that head and neck cancer patients are facing before and after radiotherapy (NAVAZESH, 1993; Chaudhury et al., 2015; Proctor, 2016). Furthermore, during such measurements, it is essential to maintain homogenous clinical conditions on every patient visit, to avoid or reduce the circadian variation. Salivary flow rate and protein composition have been shown to present the highest levels in the afternoon (Pedersen et al., 2018) which is why in this study all the samples were collected at the same hour of the day, between 13:30 and 15:30 in the dental clinic.

An advantage of unstimulated whole mouth saliva collection by passive drooling is that does not represent a technical challenge for the operator or for patients during the sample collection. This is especially important after RT because patients may develop side effects of cancer treatment, such as trismus reducing mouth opening and oral mucositis. Parotid gland stimulated saliva collection will include the use of citric acid 2% solution and the use of special instruments such as a Lashley cup that must be placed over the duct orifice, attached to the oral mucosa previously dried keeping the mouth open for 15 minutes during the collection period, all of which may be a challenge for head and neck cancer patients.

In addition to the reduction in the amount of flowing saliva, clinically a change in saliva colour and consistency at the moment of sample collection and later on when the samples were handled in the laboratory were observed subjectively, but not measured formally in this study. It would be useful to measure the rheological properties of saliva (specifically Spinnbarkeit) of head and neck cancer patients in order to analyse adhesive and elastic changes after radiotherapy, to study the association with the amount of saliva, mucin 5B and 7 protein content and dry mouth reported by patients along with the clinical changes in saliva colour and viscosity reported by clinicians.

The clinical implication of a reduced salivary flow rate after radiotherapy in the early stages is that it will hinder daily normal functions such as eating, swallowing, speaking and the use of removable prosthodontic appliances due to the lack of mucosal lubrication, hydration and dry mouth influencing negatively the patient's quality of life.

Xerostomia can affect emotional physical and social functioning of head and neck cancer survivors. Results from this study were no different, with the "dry mouth feeling" that is typically associated with RT occurring to over 90% of patients at both time points, with only 3 patients not reporting dry mouth feeling at T1, and 2 patients at T2.

However, with such a high incidence rate, one must be cautious and seek to associate findings together. One possible reasoning could be that patients experience dry mouth even when healthy, as was shown by Eliasson et al., (2009), suggesting that that the existence of areas of dry mucosa, along with decreased mucin nearby, can generate a sensation of dry mouth. Therefore, this condition can be associated to an altered protein composition, particularly mucins, resulting in dry mouth sensation even if the flow rate is not reduced(Lee et al., 2007; Dijkema et al., 2012; Chaudhury et al., 2015; Villa and Sonis, 2016; Pedersen et al., 2018; Almståhl et al., 2019).

Analysing for a potential association between xerostomia and salivary flow rate revealed that were significant associations for patients at T1. These results are in line with similar patterns found by other studies (Eisbruch et al., 1999; Wiener et al., 2010; Pedersen et al., 2018; Nguyen et al., 2018). However, this statistically significant association was no longer present at T2, with the data being statistically similar to that of baseline figures, suggesting that perhaps these are only short-term associations.

In the next chapter, these findings would be analysed regarding salivary biochemical compositional contents, to investigate levels of proteins that are involved with lubrication, such as mucins 5B and 7.

The clinical relevance of changes in saliva quantity and quality are that they directly influence lubrication of the oral cavity, hydration of the oral mucosa, alter normal mouth feel and contribute to overall oral homeostasis. With salivary flow rate established as a generally reliable measure of dry mouth, and it being shown to act as such once again in this study, one must observe associations between xerostomia, salivary flow rate and clinical observations in order to identify points of potential treatment in order to help ease patients' quality of life (from a clinical aspect) following xerostomia as a result of cancer therapy.

A few studies have assessed IMRT treatment and whether there are any associations to observe with the impacts they may have on the quality of life post-RT. There are reports in the literature based on questionnaires related directly to the quality of life regarding salivary flow rate and dry mouth feeling (Nutting et al., 2011). Nevertheless, evidence of altered salivary flow rate and protein concentration related to dry mouth feeling reported by head and neck cancer patients treated with different modalities of radiotherapy is lacking (Richards, 2014; Richards et al., 2017).

A handful of trials have collected salivary samples to measure flow rate, pH or buffering capacity for patients treated with IMRT, however, they were often of limited scope or a small number of study participants (Kwong et al., 2004; Sim et al., 2018). Furthermore, there were no other clinical assessments and there was a lack of salivary protein content analysis, specially mucins, which can make it difficult to draw conclusions as to the reasons for the underlying issues.

In contrast, Nutting et al., (2011) stated that there was no association between xerostomia and saliva flow rate in 73 head and neck cancer survivors. Twelve months after completing RT, the dry mouth feeling reported was significantly lower in patients that underwent IMRT compared to conventional RT. Although salivary flow rate was measured, the values were not presented in this study and no additional saliva analysis was included for us to compare with.

Further changes to the quality of life of patients that are attributed to IMRT, include the alterations to taste perception, with previous studies (Pow et al., 2006; Hawkins et al., 2018)

demonstrating that the use of IMRT produced oral side effects in head and neck cancer patients similar to those of traditional radiotherapy, as recorded using questionnaires by patient-reported outcomes. Similarly, this present study observed significant numbers of taste disturbances after IMRT, when compared with each patient's taste perceptions before treatment.

Taste changes were measured by individual patients reporting to be uncapable of distinguishing food flavours after radiotherapy compared to before. At T1, 65% of participants reported a reduced taste acuity, whereas at T2 the number of patients reporting changes was reduced dropping to 48%. Although this represented an improvement in taste acuity, the improvement was non-significant, with results of T2 not being statistically significantly differing from T1.

Such findings are in accordance with past studies, with Martini et al., (2019) reporting a significant taste alteration after 6 months of radiotherapy in head and neck cancer patients, when measured using the chemotherapy-induced taste alteration scale (CiTAS), On the other hand, one past study evaluating taste thresholds for each taste quality, reported that there was a recovery of taste sensitivity at one year post-radiotherapy treatment, with taste levels reaching baseline thresholds(Sandow et al., 2006). However, that study needs further review as it utilised 11 patients which may affect the results due to lack of variability in subjects. Such disparity in the findings of past studies have led Epstein et al., (2016) to conclude that the real impact of IMRT is not clear, with the possibility for taste alteration recovery after treatment to occur 6 months after therapy or the possibility for it to continue indefinitely. Furthermore, even when recovery is reported, there is a varying scale of whether the recovery is complete or partial(Oates et al., 2007; Epstein et al., 2019).

Even though the prevalence of changes in taste after cancer treatment have been described the aforementioned studies, the mechanisms involved are not well understood. There is not enough evidence of the underlying interactions between radiation and the subjective changes in taste before and after radiotherapy. It is still inconclusive whether radiation toxicity acts by affecting threshold and/or directly producing an alteration of taste perception by epithelial damage (Spotten et al., 2016; Epstein et al., 2016, 2019), or whether the taste changes perceived are a result of the reduced salivary flow rate; it has been proposed that the lack of saliva will impede the transport of molecules into taste buds receptors, as well as saliva

protection of receptor from damage will be reduced(Epstein and Barasch, 2010; Epstein et al., 2016).

This theory has been explored further by the Epstein research group, who have stated that that taste buds acuity requires an appropriate volume of saliva in the mouth, and with almost all radiotherapy studies observing a reduced salivary flow rate, including this study observing a reduced salivary flow rate in patients at both T1 (6 months) and T2 (12 months) and a significant and negative association between this reduction and taste variation reported by patients (Table 2.9), they conclude that this must be cause of taste alterations with the lack of saliva has been causing an incapacity of dissolving food molecules and transport these in to taste buds (Epstein and Barasch, 2010; Epstein et al., 2012, 2016, 2019).

Apart from these changes, taste perception may also be a direct result of the damage on the epithelial cells caused by radiotherapy, which in this case has been related to oral mucositis onset during radiotherapy (Ruo Redda and Allis, 2006; Epstein and Barasch, 2010; Irune et al., 2014). Epithelial toxicity caused by radiotherapy has been associated with taste changes in some past studies, along with the resultant mucosal damage(J. B. Epstein et al., 2002; Doty et al., 2017). Furthermore, Ogama et al., (2010) found associations between reduced salivary flow rate, oral mucositis and taste in head and neck cancer patients treated with radiotherapy.

It is especially important to keep in mind that although these changes are significant, in all cases they are self-reported by patients, causing the data to be subjective. Therefore, this represents a limitation regarding taste variation and dry mouth reported by patients by using one question extracted from a full questionnaire (Fox et al., (1987) and LENT-SOMA questionnaire) that explore different dimensions of these two factors regarding radiotherapy side effects. Moreover, it was selected only one question from each survey which reduced the scope of the information that can be obtained, along with the division of patients in two groups with or without the condition only, without taking in account the severity of the condition and the possible differences during specific times of the day, or the possible relation to certain daily functional activities. Therefore, this data represents a first approximation regarding xerostomia and taste acuity in this group of patients, in order to associate with objective measurements of hyposalivation and salivary composition regarding mouth feel and taste after IMRT.

Oral mucositis presence and severity was reported by the oncology team during and after radiotherapy and were in concordance with the results reported by Van Gestel et al., (2011). It was observed that there were 31 patients that developed mucositis during radiotherapy and the maximum grade at any time point was grade 3 representing 40% of the patients in concordance with Orlandi et al., (2018) reported severe acute OM (G≥3) during IMRT using the Common Terminology Criteria for Adverse Events (CTCAE) scale version 4.03. On the other hand, Van Gestel et al., (2011), using the RTOG scale reported grade 3 oral mucositis in 41 head and neck cancer patients treated with IMRT, representing 85% of the total number of subjects studied which is twice as much that what it was reported in this study. A drawback in oral mucositis research is the lack of a universal scale, there is not an objective and standardised tool to assess oral mucositis severity in the clinic. There is available a high number of instruments validated, these scoring systems are subjective based on patient experience related to pain and functional disability, disregarding anatomy, size of ulcers and regions affected thorough the oral cavity. Therefore, mucosal evaluation outcomes mainly depend on physician clinical experience and training in order to define the categories in an effort to increase inter observer reliability.

There are contradictory opinions among researchers based on these grading systems some of them will choose one of the existing others developed their own in order to standardize, validate and remove subjective observations from their research outcomes. There is no consensus regarding these evaluation instruments (Sonis et al., 2001). There is no universal validated scale and no evidence of any superiority among system to report oral mucositis rates. However, the WHO toxicity scale has been used since 1979 more than 40 years (Quinn et al., 2008).

After radiotherapy the number of patients presenting oral mucositis were reduced at T1 and T2 reaching 16% and 9% respectively, similarly after RT persistent mucositis was seen in 19.2% in a previous report developed by Jham et al., (2008) using the WHO scale patients were followed by 3 months after the end of RT. These past studies have established there is a risk of radiation-induced mucositis that varies with the site of radiotherapy volume of irradiated tissue, dosage, and fractionation (Chao et al., 2001; Pinna et al., 2015; Klein et al., 2014; Laheij et al., 2015; Sroussi et al., 2017).

This chapter's results showed no difference among different tumour locations regarding oral mucositis development. It has been stablished in the literature that hypopharyngeal or laryngeal cancers have less risk of developing oral mucositis than patients with cancers of the oral cavity (Sonis, 2009). In this group, oral mucositis severity was not significantly different among distinct tumour location, despite the fact that the number of patients conforming each group were not equal, in almost every group there were patients reaching grade 2 and grade 3 severity. Therefore, it can be concluded that the location of the primary tumour was not a factor of difference in severity and onset, as well as regarding IMRT dosage and fractioning scheme, these were no dissimilar (62.5 Gy mean dose). An explanation for this situation could be related to the patient's oral conditions in response to IMRT effects(Villa and Sonis, 2016). It has been shown in reports that good oral hygiene and proper oral/dental health care before IMRT might help to reduce duration severity and even the risk of oral mucositis. Another factor is the microorganisms that are capable to colonize ulcers contributing to oral mucositis, this bacterial shift is led by the lack of protective salivary functions due to the reduced flow rate and altered composition of proteins related to antibacterial protection. Age is another risk factor, older patients are more prone of developing mucositis, in this group the mean age was 62 years with no statistical difference among subjects(Sciubba and Goldenberg, 2006; Villa and Sonis, 2016).

This study revealed significant associations at T1 & T2 between oral mucositis and reduced salivary flow rate in all subjects. There were also significant associations between the dry mouth feeling reported by patients and oral mucositis at T1 and T2, showing a connection between these side effects and salivary gland dysfunction after IMRT. These associations report the oral environment influence, specially the role of saliva in oral mucositis course and severity regarding mucosal wetting, lubrication and bacterial protective functions and colonization (Sonis, 2009).

Past studies have proposed that there could be a relationship between salivary gland dysfunction and elevated caries prevalence (Mese and Matsuo, 2007; Dawes et al., 2015; Pinna et al., 2015; Gao et al., 2016). When observing caries presence in the current study, it was found that carious lesion development was decreased significantly at both T1 and T2, in comparison to pre-IMRT. Similarly, when measuring the number of surfaces affected by caries, it was also observed that carious lesion development was decreased significantly at

both T1 and T2. This is keeping in line with past studies, such as Duarte at al. (2014) following neck and neck cancer patients every 90 days for 1 year, who concluded that both IMRT and conventional radiotherapy resulted in a lower number of carious lesions after treatment.

However, this is not to suggest that IMRT / RT had halted the development of carious lesions, but rather, when taken in context with the number of teeth remaining (Figure 2.4), it is concluded that the teeth that were in the high dose radiation beam that presented with carious lesions that were considered non restorable or if they required significant restorative treatment, endodontic procedure were extracted immediately prior to radiotherapy treatment (Hancock et al., 2003), which can also be shown by measurements of number of teeth missing at T1 and T2 (Figure 2.5). Therefore, that is main reason why the number of teeth missed index was significantly increased at T1 and T2 in comparison to T0. A standard protocol exists to extract any teeth that present with carious lesions, active periapical lesions, extensive and chronic periapical lesions and unrestorable teeth, prior to radiotherapy (Joshi, 2010) - ideally 3 weeks or more prior to allow time for healing before radiotherapy begins (Makkonen et al., 1987).

If the study were to have gone on for an extended period of time, it is to be expected, based on the past literature, that caries incidence would have returned to baseline levels within 2 years, as studies have shown carious lesions redevelop 2 years after radiotherapy (Schuurhuis et al., 2011). This is because one of the factors that affects dental status is time elapsed following radiotherapy, with every month following the end of radiotherapy only increasing the odds of further tooth damage(Walker et al., 2011). However, one mitigating factor that might have contributed to the prevention of lesions developing within 2 years, is the daily fluoride treatment participants (Duraphat® 5000, twice per day) in this study, as recommended by the PATIENTS' dental consultant at Guy's hospital. Fluoride treatment has been shown to mitigate risks of more severe radiation caries (Andrews and Griffiths, 2001, Pitts et al 2017). Risk of severe radiation caries has been linked to salivary gland dysfunction, as measured by salivary flow rate. It should be noted that past studies have observed no single salivary parameter increases susceptibility to dental caries, and that several parameters should be taken into consideration in combination with each other, alongside other non-quantitative factors such as socio-demographic, behavioural and clinical aspects (Gao et al., 2016).

Initiation and progression of caries lesions are a result of unbalance between protective and pathological factors present in the mouth; protective factors stimulate remineralization while pathological factors will make the disease progress. (Pitts et al 2017; Featherstone et al 2018;). In order to increase protective factors, present in the early clinical stages of caries, all patients under the care of the Special Care Dentistry Unit are prescribed daily use of sodium fluoride toothpaste Duraphat 5000 (twice per day) during the pre IMRT oral examination (RCS guidelines 2018). This high fluoride protocol continues indefinitely with the aim of reducing the frequency of complication such as radiotherapy caries (Pitts et al 2017; Pedersen et al 2018). Patient compliance with this protocol is reviewed during the post radiotherapy T1 (6 months) assessment. In addition, participants had an extra assessment post IMRT at T2, which have assisted with continued adherence to this regime, as shown by the decreased number of carious lesion development at both T1 and T2. However, the number of teeth extracted or missing post-radiotherapy (a mean of 13.5 teeth at both T1 & T2) represents the high risk of becoming edentulous in the long term, affecting daily functions such as mastication and eating. This presents another severe challenge for oral rehabilitation and one not helped by the reduced salivary flow rate.

It is important to remark that the oral evaluation was conducted at each visit by the same calibrated clinical examiner (specialist dentist) in order to identify adverse oral and dental outcomes, through an appropriate diagnosis, being capable of differentiating tooth pain from ulcerative oral mucositis pain by exhaustive facial and intra-oral examinations at every time point. Additionally, at T1 and T2 oral mucositis intensity was grade 1, as characterised by mild clinical symptoms including erythema, without ulcerative lesions or pain.

The reduced salivary flow rate appeared to be negatively and significantly associated with all the clinical parameters related to the dental assessment (DMFT), after radiotherapy at both T1 and T2. This is in keeping with the past studies that have shown associations between reduced salivary flow rates in cancer patients and with increased risk of caries or candida infections(Frenkel and Ribbeck, 2015; Gao et al., 2016; Pitts et al., 2017). These diseases would have occurred in those studies as saliva is vital in maintaining mineral equilibrium on enamel surfaces, providing the ions required for remineralization along with maintaining bacterial equilibrium (Pitts et al., 2017).

Salivary flow rate and composition plays a critical role in the oral ecosystem symbiosis and biofilm formation. In this study participants presented with a significantly reduced salivary flow rate. During radiotherapy lack of saliva would have resulted in soft tissue inflammation due to a reduced lubrication and barrier function. This would have been combined with the direct effect of radiotherapy on epithelial cells causing toxicity leading to a cell death and ulcers as well as a shift in the diversity of oral biofilm population disrupting normal oral homeostasis and promoting pathogenic bacteria growth (Kielbassa et al., 2006; Jham et al., 2008; Gao et al., 2014; Liu et al., 2014; Xu et al., 2014; Deng et al., 2015; Pinna et al., 2015).

Participants with hyposalivation caused by radiation therapy showed differences in oral microflora composition compared with age-gender control patients (Almstahl et al 2001), which included an increased susceptibility to bacterial and yeast infections (Chaudhury et al 2015). However, participants with hyposalivation presented with similar number of tooth surfaces covered with plaque compared with controls. The difference in bacterial composition was associated with an altered salivary protein concentration (Almstahl et al 2001), reduced salivary flow rate and poor oral hygiene which meant increased risks of an unbalanced oral microbiome mainly dominated by the microorganisms associated with oral diseases (Killian et al 2016).Therefore, it would be imperative to assess and monitoring the periodontal status of patients before and after radiotherapy given the possible association between bacterial periodontitis and oral mucositis severity and duration (Laheij et al 2014; Maria et al 2017). Furthermore, the presence of ulcers results in a natural barrier breach providing an open entry for microorganism and cytokines to the underlying tissues thus increasing the risk for secondary infections such as bacteraemia and sepsis (Laheij et al 2014; Villa & Sonis 2015,2016; De sanctis 2016). It has been observed that during and after radiotherapy head and neck cancer patients can suffer severe mucositis (Sonis 2004; Vasconcelos et al., 2016; Richards et al., 2017) along with an altered microbial diversity, particularly an increased number of bacteria associated with periodontitis. Gram-negative anaerobic bacteria and other periodontal pathogens such as *Porphyromonas gingivalis, Fusobacterium* and *Prevotella* are predominantly associated with the onset of ulcerative oral mucositis and impact the severity. This suggests that their abundance might represent a risk factor for aggravation of ulcers along with a delay in mucosal healing. (Laheij et al.2012; Laheij et al 2014; Zhu et al., 2017; Elerson et al 2018; Laheij et al., 2020). However, the role of these

bacteria regarding host microbe interaction in promoting or modifying inflammation and exacerbating oral mucositis progression needs to be confirmed and elucidated in future studies. This in turn will inform the need for periodontal routine screening in this group of patients (Sonis et al 2004 etc Laheij et al.2012; Laheij et al 2014; Zhu et al., 2017; Elerson et al 2018; Laheij et al., 2020).

Overall, this study concludes with several findings, starting with the fact that salivary flow showed a significant, transient reduction in quantity following IMRT. This was in line with past studies that found IMRT to not fully spare tissues surrounding target treatment area, when measured in terms of salivary flow rate(Dijkema et al., 2008), xerostomia (Dijkema et al., 2010), and other oral symptoms such as hyposalivation in general (Nutting et al., 2011; Nguyen et al., 2018). With over 90% of patients in this study exhibiting signs of and reporting dry mouth, the intensity modulated radiotherapy treatment clearly lead to a vulnerable oral environment and disrupted oral homeostasis, leading to deteriorated clinical outcomes, such as reduced number of teeth, potential edentulism, all of which was statistically significantly associated to the original finding of reduced salivary flow rate. There are significant associations between salivary flow rate and dry mouth feeling, taste alterations oral mucositis assessment, number of teeth present, number of teeth missing and number of carious lesions (as measured by both teeth and surfaces). These findings corroborate that salivary flow rate is an objective measure of recording salivary gland damage (hyposalivation), which clinically is expressed as all of aforementioned side effects.

However, the clinical relevance of such findings is dependent on what particular changes are occurring within the remaining saliva, dependent on the function of proteins that make up the composition of saliva (Jawad et al., 2015). Therefore, perhaps the next study should begin to analyse the bio-composition of saliva in order to evaluate the effects of IMRT on salivary gland output, allowing for conclusions as to whether it is definitively the reduced flow rate that is affecting dental health or whether it is the altered compositions of the remaining saliva. So far, this has all reinforced the past studies that found IMRT does not fully spare tissues that surround the sites of targeted radiotherapy treatment, when measured as xerostomia or symptoms (Dijkema et al., 2010; Nutting et al., 2011). However, the direct causalities for how IMRT affect salivary gland functions is as of yet unclear, and therefore the analysis of how IMRT affects particular salivary proteins is explored further in Chapter 3.

Chapter 3 Impact of Radiotherapy on The Biochemical Composition of UWMS

3.1 Introduction

Chapter 2 detailed how salivary flow rate was decreased significantly following IMRT, correlating with an increase in the reporting of xerostomia and taste changes, as well as a significant reduction in number of teeth present in the mouth post-radiotherapy and variations in the quantity of carious lesions at each time point.

Previous studies have observed changes in the biochemical composition of saliva following both traditional therapy (Roesink et al., 2001) as well as IMRT (Richards et al., 2017), however there remain many unanswered questions, such as the long-term nature of the changes in the protein composition as well as whether there are associations between individual salivary proteins and their corresponding xerostomia reporting, taste acuity, carious lesion incidence, and mucositis severity.

This chapter analyses the changes of individual salivary possible molecular markers in UWMS composition, in order to assess major salivary gland function through assessing total protein concentration and secretion rate, as well as specific proteins associated with each gland.

Analysis was performed on UWMS of 40 patients, observing organic salivary composition biomarker that indicate various oral functions. UWMS is a mixture of all salivary gland outputs and this analysis concerned proteins and glycoproteins expression with specific functions that are directly linked to the side effects of radiotherapy reported in Chapter 3.

The Mucins investigated were mucin 5B and 7. Their central function is lubrication and hydration of oral mucosa due to their water binding capacity. Lack of these glycoproteins or alteration in their composition is associated with dry mouth feeling(Eliasson et al., 2005; Vijay et al., 2015; Proctor, 2016; Pedersen et al., 2018). It has been shown that modifications in salivary mucin 5B and mucin 7 affects mucosal surfaces due to reduced salivary wetting capability and altered lubrication. Mucosal surfaces become dry and atrophic, thus more prone to injury (Vissink et al., 2010).

In addition, mucin 5B and mucin 7 form a protective barrier preventing erosion, abrasion and ulceration during daily oral functions such as mastication, swallowing and speaking (Vissink et al., 2010; Carpenter, 2013b; H. L. Gibbins et al., 2014; Dawes et al., 2015; Proctor, 2016; Pedersen et al., 2018). Mucosal integrity and protection are vital in HNC patients due to the high rate of oral mucositis onset during radiotherapy. Therefore, it is important to asses concentration of these salivary proteins as well as their functionality(Dawes et al., 2015). It has been suggested by Sonis, (2004) that during the formative stages of ulcers the amount of saliva decreases whilst mucositis progresses (Tschoppe et al., 2010; Pinna et al., 2015).

It is well known that mucin 5B and mucin 7 are the main constituents of mucosal pellicle, along with IgA, forming a complex with mucin 7 (Vissink et al., 2010; H. L. Gibbins et al., 2014; Dawes et al., 2015). IgA is the main antibody in saliva and plays an important role in bacterial colonization, mucosal protection, defence against microorganism, mucosal immune system and mucosal pellicle forming and regulating an epithelial barrier (Brandtzaeg, 2009; Dawes et al., 2015; Hemadi et al., 2017; Lynge Pedersen and Belstrøm, 2019). IgA is secreted by B Lymphocytes in salivary glands including minor glands and parotid glands (Crawford et al., 1975; Brandtzaeg, 2009, 2013; Lynge Pedersen and Belstrøm, 2019).

α-Amylase is one of the most prevalent enzymes in saliva and is generally considered to be a reliable marker of serous cell function and can therefore act as a marker for parotid gland function. Past studies have shown α-amylase concentration levels can be linked to protection against caries (Dawes 2008, Borghi et al., 2017).

Albumin concentration is also considered an important marker for potential mucositis, as an increased concentration is related to an inflammatory process and increased vascular permeability (Jensen et al., 2003). Past studies have also observed that albumin, statherin, cystatin, proline rich protein (PRP), mucins 5B and 7 form acquired enamel pellicle, reducing the possibility of erosion and abrasion, determining biofilm formation, keeping mineral equilibrium by maintaining a high concentration of calcium and buffer capacity (H. L. Gibbins et al., 2014; Hannig et al., 2017).

Cystatin S is a protein related to enamel pellicle formation which has antibacterial functions, such as inhibiting the action of endogenous, bacterial and parasitic protozoan proteases, or binding bacterial lipopolysaccharides(Messana et al., 2008; Magister and Kos, 2013; Hemadi

et al., 2017). The cystatins family of proteins are also part of the acquired enamel pellicle formation and play an important role in remineralisation process, as well as taste functions (Gao et al., 2016; Picco et al., 2017; Hemadi et al., 2017). Cystatin S may be considered a biomarker in assessing submandibular glands function(Martini et al., 2017).

Proline rich proteins are secreted by parotid and submandibular glands and contain between 25-42% of proline amino-acids which affects this protein structure (Carpenter, 2013b). Acidic and basic proline rich proteins are part of the acquired enamel pellicle due to their calcium hydroxide binding properties. Proline rich proteins, statherin and other phosphor containing proteins have calcium binding properties, thereby maintaining a higher concentration of calcium than teeth (supersaturated saliva), in order to prevent dissolution of teeth and to avoid their precipitation, especially in the cervical margin causing calculus (Carpenter, 2013b; Hemadi et al., 2017). These proteins participate in oral bacterial clearance, by binding bacteria, fungi and viruses towards the stomach. Furthermore, these proteins have a polyphenol binding affinity, acting as polyphenol precipitators (tannins included) in the perception of astringency (Fábián et al., 2015).

Statherin is a phosphoprotein secreted from parotid, submandibular and sublingual glands, which plays a multifunctional role in the oral cavity; maintaining calcium homeostasis, preventing its precipitation and crystal growth, as well as maintaining saliva supersaturation in order to stimulate the remineralisation process.

Carbonic Anhydrase VI (CA VI) is an enzyme secreted by parotid and submandibular glands that plays a fundamental role in controlling taste sensation and taste bud growth, by facilitating the interaction between food particles and taste buds (Denny et al., 2008, Welton 2012). CA VI is activated in order to counteract the excessive acidity of the tooth surface biofilm by catalysing the most important buffer reaction in the oral environment; the carbonic dioxide equilibrium in saliva (Öztürk et al., 2008; Frasseto et al., 2012; Zwier et al., 2013; Cardoso et al., 2017; Pedersen et al., 2018), in order to increase bicarbonate salivary levels especially with acidic food. Moreover, this buffer mechanism plays a role in reducing tooth demineralisation by neutralising acidic pH of biofilm (Kimoto et al., 2006; Cardoso et al., 2017).

In previous chapters of this thesis was shown in detail how salivary flow rate was decreased statistically significantly following IMRT at two time points, along with over 90% of the patients reporting xerostomia and taste changes, as well as a deteriorate dental clinical outcomes such as a significant reduction in number of teeth present in the mouth post-radiotherapy. Moreover, it was demonstrated the links among dental assessments, dry mouth perception, taste perception and oral mucositis to a reduced UWMS flow rate following IMRT. Regarding salivary flow rate past studies observed that IMRT not fully spare area that surround tumour (Dijkema et al., 2008). In the same pattern Dijkema et al., (2010); Nutting et al., (2011); Nguyen et al., (2018) reported xerostomia, and other oral symptoms such as hyposalivation in general in patients treated with IMRT.

The clinical relevance of protein composition variation after IMRT is dependant of the function of each protein (Chao et al., 2001; Jawad et al., 2015). Whilst it is important to study and identify the possible association between these clinical side effects that are affecting patient's quality of life with the quality of UWM saliva in order to find a target that allow to explain its participation in onset of these side effects. Past studies have showed some of these biochemical changes in saliva concentration and salivary flow reduction post radiotherapy (Dijkema et al., 2012).

It has been proposed by some authors that a reduced salivary flow rate is usually accompanied by an altered saliva composition (H. L. Gibbins et al., 2014; Hannah L. Gibbins et al., 2014; Hannig et al., 2017), the association between these proteins and salivary flow rate is still not clear specially after radiotherapy and adding the longitudinal factor including different time point.

The understanding of the association between oral side effects due to radiotherapy and salivary protein composition alteration is not yet fully analysed in the literature, some authors have attempted to assess the interrelation between radiothera

py side effects (Wijers et al., 2002; Nutting et al., 2011; Randall et al., 2013; Belstrøm et al., 2016; Janus et al., 2017), showing the correlation between a reduction in salivary flow rate and the salivary gland hypofunction after cancer treatment, despite the use of IMRT and the current preventive measures given by the oncology and dental team in the UK(Walker et al., 2011; De Siqueira Mellara et al., 2014; Lieshout and Bots, 2014; Laheij et al., 2015). Others

studies have informed prevalence of xerostomia and taste altered perception following HNC (Ruo Redda and Allis, 2006; Epstein and Barasch, 2010; Epstein et al., 2016; Spotten et al., 2016; Epstein et al., 2019) and the link of these symptoms with reduced salivary flow rate (Epstein et al., 2019).

There is a lack of research that evaluate the role of proteins regarding lubrication and mucosal protection during and after RT (Randall et al., 2013). The absence of studies that have evaluated the role of mucin 5B in xerostomia onset and development is an impetus for this analysis.

This chapter analyses the changes of the nine aforementioned individual salivary biomarkers in UWMS composition of 40 patients in order to evaluate major salivary gland function throughout, whilst assessing total protein concentration and secretion rate, as well as specific proteins associated with each gland. In addition, In this chapter it assessed the possible association between the altered clinical outcomes reported in chapter 2 and the salivary compositional changes in order to determinate the dependence between these clinical and biochemical factors, regarding proteins functions that make up the composition of saliva (Jawad et al., 2015).

3.2 Aims

The aim of this longitudinal trial was to assess salivary gland function of HNC patients pre (T0) and post IMRT (T1 and 2) by analysing protein concentration and secretion rate and investigate the possible associations between these proteins and clinical outcomes.

Null hypothesis: The hypothesis for this chapter is that IMRT will not affect the protein composition and secretion rate. There is no association between salivary proteins and oral side effects of radiotherapy.

Objectives

- To assess the effect of IMRT on salivary protein concentration and secretion rate of UWMS in HNC patients at different time points (T0 and compared to T1 and T2)
- To analyse the possible associations between proteins concentration and secretion rate with salivary flow rate, self-reported xerostomia and self-reported taste changes by patients.

- To determine whether these proteins analysed have role in these clinical side effects expression regarding salivary gland function particular production involvement in UWMS.

3.3 Material and Methods

3.3.1 Patient Recruitment

Forty HNC patients were recruited in line with the Helsinki declaration with full, written informed consent gained. The study was approved by the North of Scotland Research Ethics Service (NRES) Committee foundation in October 2016 (16/NS/0116), the Health Research Authority NHS (IRAS Project ID:199100) and the patient recruitment letter is appended (Appendix no.1).

Recruitment was done prior to IMRT (T0) at Guy's Hospital London in the Special Dental Care Unit. Patients were seen at six months post IMRT (T1) and 12 months post IMRT (T2). 40 patients were recruited at T0, 38 patients were seen at T1 and 34 patients at T2 respectively.

3.3.2 Saliva Sample Collection

All saliva samples were collected from the patients by drooling method at every time point, and flow rate was assessed as described in chapter 2. One hundred and eleven saliva samples were collected and analysed.

3.3.3 Sample Analysis

Samples were analysed for total protein content using the Bicinchoninic Acid Assay (BCA), targeted protein concentration by Enzyme-Linked Immunosorbent Assay (ELISA), Periodic Acid Schiff (PAS) staining, Coomassie Brilliant blue and α-amylase activity by the standard kinetic enzyme assay (Salimetrics).

Prior to biochemical analysis UWMS samples were centrifuged at 10000 RPM for 5 mins at 4°C. The supernatant was separated to be used to analyse protein content and the pellet was preserved to perform DNA extraction.

3.3.3.1 Protein Analysis Concentration and Secretion Rate

In order to determine the salivary protein composition, the total protein content /secretion rate, specific protein concentrations and their secretion rates were analysed. Nine proteins (mucin 5B mucin 7, IgA, α-amylase, albumin carbonic anhydrase VI, acidic proline rich protein and statherin) were selected due to their relationship to host defence, antimicrobial, bacterial adherence, colonization, mucosal pellicle, enamel pellicle, viscoelastic, rheological properties, lubrication, and remineralization.

In summary mucin 5B mucin 7, IgA, α-amylase, albumin carbonic anhydrase VI, acidic proline rich protein and statherin all salivary proteins related to caries development, xerostomia, taste alteration and oral mucositis before and after radiation therapy in a longitudinal clinical study (12th months) were analysed.

3.3.3.2 Total Protein Concentration (TPC)

Total protein concentration of UWMS was determined using the BCA protein assay (Thermo fisher scientific, IL, USA), in a 96 multi-well plate according to manufacturer's instructions. Results were derived from a standard curve generated using Bovine Serum Albumin (BSA) serial dilution incubated at the same time and protein concentrations were determined. UWMS samples were assessed at a dilution of 1:10 in ultra-high-quality water. The absorbance was measured at 540nm using a plate reader (iMark Microplate Absorbance reader BIORAD, UK). All the saliva samples were analysed in duplicate. Total protein concentration was expressed in mg/ml.

3.3.3.3 Total Protein Secretion Rate

Protein secretion rate was calculated to assess salivary gland function at every time point (pre and post radiotherapy) including the salivary flow rate (ml/min) and the total protein concentration per ml of saliva. Total protein secretion rate was calculated by multiplication of the salivary flow rate and protein concentration in ug / min (Carpenter et al., 2000).

3.3.3.4 Sample Preparation and Gel Electrophoresis

All samples were prepared under the same conditions, adding 25% NuPage lithium dodecyl sulphate sample buffer (LDS, Invitrogen), 10% 0.5 dithiothreitol (DTT) and boiled for 3 min at 100 ºC. UWMS samples were analysed for protein content using sodium duodecil sulphate polyacrylamide gel electrophoresis (SDS-PAGE). NuPage Novex, 4-12% bis tris gels Invitrogen were used to assess 2-200 KDa molecular weight proteins. The gels were set up in a Xcell vertical electrophoresis unit (Invitrogen, Thermo Fisher Scientific, UK) with 25 ml of NuPage MES SDS running buffer (x20 stock) (Invitrogen, Thermo Fisher Scientific, UK) and 475 ml double distilled water (dd H_2O)Total protein concentration of samples was measured using the BCA assay and the protein load was adjusted to 20µg per lane. 5 µl of SeeBlue Plus Pre-stained marker (Invitrogen, Thermo Fisher Scientific, UK) was loaded in the first and last lanes. The samples underwent electrophoresis for 35 minutes at 125 mA and 200 volts constant.

3.3.3.5 Mucin Detection and Quantification

For the purpose of determining the effect of irradiation on mucin 5B and mucin 7 concentration in UWMS, saliva samples were electrophoriesed, and the gels were stained with PAS stain for the detection of mucin 5B and mucin 7.

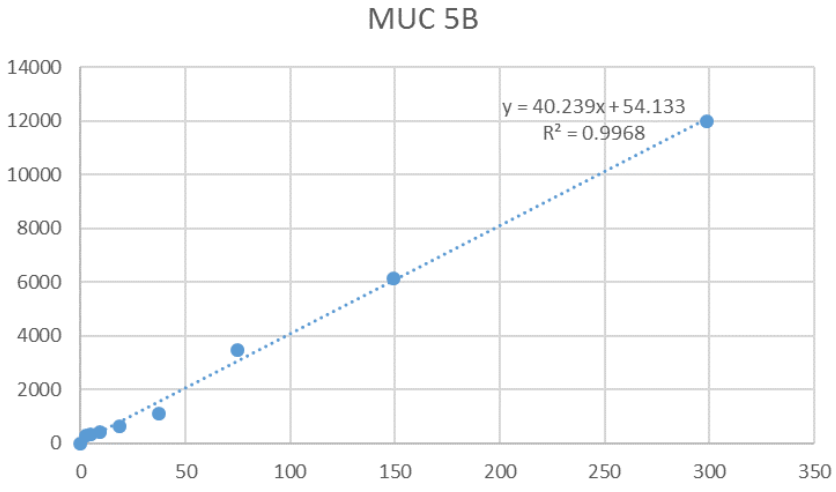
3.3.3.5.1 Periodic Acid Schiff Staining of Protein Gels

PAS was used to detect and assess glycoproteins. After electrophoresis, gels were fixed in a fixing solution 25% methanol and 10% glacial acetic acid for 60 minutes and then washed in UHQ water for 20 minutes and incubated in 2% (w/v) periodic acid (sigma) for 15 minutes.

Subsequently, gels were rinsed with UHQ water for 4 min in total and using Schiff reagent (VWR, Leicestershire, UK), gels were incubated in the dark with gentle agitation for up to 60 minutes or until pink stained bands appeared. The gels were de-stained in water and scanned on an automated image developing system, the ChemiDoc (Bio-Rad, Hemel Hempstead, UK) in order to identify mucin glycoproteins.

3.3.3.5.2 Mucin Semi-quantification

MUC5B and MUC7 mucin glycoproteins were semi-quantified using purified mucin fractions of known concentrations (Malmo University, Sweden). The purified mucin fractions were serially diluted, underwent electrophoresis and stained with PAS. Images of the PAS stained gel were analysed for band intensity using the ChemiDoc. The bands were converted to peaks and the area under each curve gave the pixel intensity. Standard curves and linear equations were generated for mucins (MUC5b and MUC7) concentration against pixel intensity. The mucin concentrations of the samples were calculated from the known pixel intensities using the linear equation obtained from the standard curve shown in Figure 3.1.



(A)

(B)

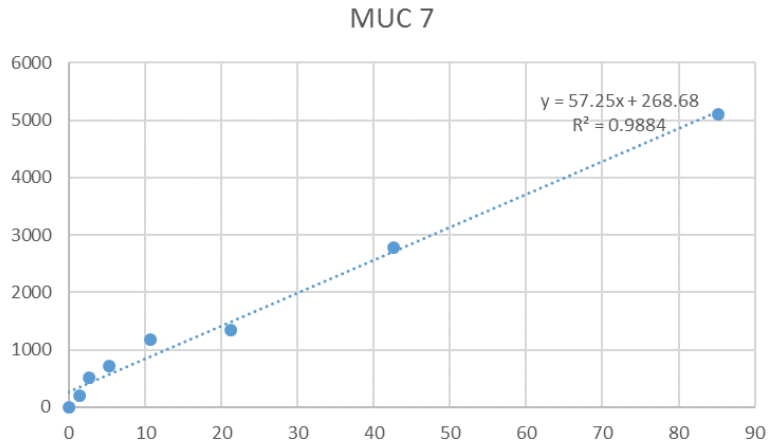


Figure 3.1 Mucin standard-concentration curves for (A) mucin 5B (MUC 5B), (B) mucin 7 (MUC 7).

Purified mucins with their linear equations used to calculate the concentration of MUC5B and MUC7 in the different test samples.

3.3.3.6 α-Amylase Kinetic Assay

Detection of α-amylase concentration in UWMS samples was carried out and analysed by measuring the enzymatic activity of this protein expressed in units.

α-Amylase activity was used to determine its concentration in units in saliva samples using the α-amylase assay (amylase kinetic assay, Sialimetrics LLC, PA, USA). UWMS samples were diluted with α-amylase diluent (1:200). Standard and diluted saliva samples (8µL) were added to each well of 96 well microtitre plate. The α-amylase substrate was heated at 37ºC for 20 minutes and 320 µL of the α-amylase substrate was added to each well. The absorbance was measured at 405 nm at two time points one minute, then again two minutes later. The calculations were carried out following manufacturer's instructions.

3.3.3.7 ELISA Protein Detection

IgA, albumin, cystatin S, proline rich protein and statherin concentration in UWMS were determined using ELISA. Quantification of these proteins was performed using a colorimetric reaction by adding a substrate chromogen reagent to a labelled enzyme to visualize in a

spectrophotometer in a 96 well-plate for each assay which permitted to evaluate a higher number of patients per assay, an advantage aspect to other available tests (Almsthal et a.,l 2001; Dijkema et al., 2012, Thermo Fisher Scientific Hand book 2015, Glasgow, UK).

3.3.3.7.1 IgA ELISA

Concentration of IgA in UWMS was assessed using ELISA (Sandwich kit, Cusabio Biotech USA). The ELISA microplate was pre-coated with IgA capture antibody. Samples were diluted in duplicate along with the standard and incubated at 37ºC for two hours followed by removing the liquid of each well without washing. Biotinylated mouse anti-human serum IgA detection antibody diluted to the working concentration was added and incubated at 37ºC for 1 hour followed by three washes. Streptavidin conjugated to horseradish peroxidase was added for 1 hour at 37ºC and followed by five final washes. Substrate solution consisting in a mixture (1:1) of $H_2O_2$and tetramethylbenzidine was added for 20 minutes at 37ºC. The reaction was stopped with 2M sulphuric acid and the absorbance was read within 5 minutes at 450 - 540nm to correct optical imperfections in the plate.

Human IgA sensitivity was 0.24 ng/mL and the detection range were 0.24 - 1000 ng/ml.

3.3.3.7.2 Albumin ELISA

Concentration of albumin in UWMS samples was measured using ELISA (Duo Set Elisa R&D Systems, Minneapolis, USA)

Mouse anti-human serum albumin capture antibody reconstituted with phosphate buffered saline (PBS) and diluted to the working concentration of 2ug/ml, was used to coat the ELISA microtitre plates (Thermo Scientific, UK) overnight. Three washes in phosphate buffered saline with 1% Tween (PBS-T) pH 7.2 were completed. The ELISA plates were blocked with 1% BSA in PBS pH 7.2 for 1 hour followed by three further PBS-T washes. Samples were diluted in duplicate along with the standard and incubated at room temperature and pressure for 2 hours followed by 3 PBS-T washes. Biotinylated mouse anti-human serum albumin detection antibody diluted with 1% BSA in PBS to the working concentration of 125ng/ml, was added

and incubated at room temperature for 2 hours followed by 3 PBS-T washes. Streptavidin conjugated to horseradish peroxidase diluted with 1% BSA in PBS was added for 20 minutes at room temperature followed by 3 final PBS-T washes. Substrate solution was added consisting in a mixture (1:1) of H_2O_2 and tetramethylbenzidine. The reaction was stopped with 2M sulphuric acid and the absorbance was read at 450nm and 540nm to correct optical imperfections in the plate.

3.3.3.7.3 Cystatin S ELISA

Concentration of cystatin S in UWMS was assessed using ELISA (CST4, Sandwich Cloud Clone Corp., USA). Cystatin S (CST4) capture antibody was pre-coated onto the ELISA microplate. Samples were diluted in duplicate along with the standard and incubated at 37C for 1 hour followed by removing the liquid of each well without washing. Biotinylated mouse anti human serum cystatin S detection antibody diluted to the working concentration was added and incubated at 37ºC for 1 hour followed by three washes. Streptavidin conjugated to horseradish peroxidase was added for 30 minutes at 37ºC and followed by five final washes. Substrate solution consisting in a mixture (1:1) of H_2O_2 and tetramethylbenzidine was added for 20 minutes at 37ºC**.** The reaction was stopped with 2M sulphuric acid and the absorbance was read within 5 minutes at 450nm. Detection range 0.156 - 10ng/ml, sensitivity <0.066ng/ml.

3.3.3.7.4 Proline Rich Protein ELISA

Concentration of Proline Rich Protein (PRP) in UWMS was assessed using ELISA (Sandwich Kit Thermo-scientific Pierce, USA).

The ELISA microplate was pre-coated with a Human uPA (PRAP1) capture antibody. Samples were diluted in duplicate along with the standard and incubated at room temperature for 2.5 hours followed by four washes. Biotinylated mouse anti human serum PRP detection antibody diluted to the working concentration was added and incubated at room temperature for 1 hour followed by four washes. Streptavidin conjugated to horseradish peroxidase was added

for 45 minutes at room temperature and followed by four final washes. Substrate solution consisting in a mixture (1:1) of H_2O_2 and tetramethylbenzidine was added for 30 minutes at room temperature. The reaction was stopped with 2M sulphuric acid and the absorbance was read within 5-10 minutes at 450 - 540nm to correct optical imperfections in the plate. Elisa sensitivity 10pg/ml.

3.3.3.7.5 Statherin ELISA

Concentration of Statherin in UWMS was assessed using ELISA (Sandwich kit Cusabio Biotech USA). Statherin capture antibody was pre-coated onto the ELISA microplate. Samples were diluted in duplicate along with the standard and incubated at 37 ºC for two hours followed by removing the liquid of each well without a wash. Biotinylated mouse anti-human serum statherin detection antibody diluted to the working concentration was added and incubated at 37ºC for 1 hour followed by three washes. Streptavidin conjugated to horseradish peroxidase was added for 1hour at 37ºC and followed by five final washes. Substrate solution consisting in a mixture (1:1) of H_2O_2 and tetramethylbenzidine was added for 20 minutes at 37ºC. The reaction was stopped with 2M sulphuric acid and the absorbance was read at 450nm and 540nm to correct optical imperfections in the plate.

The Detection range of this ELISA was 78 - 5000 ng/mL and the sensitivity was 19.5 ng/mL.

3.3.3.8 Coomassie Brilliant blue (R250)

Coomassie stain was used for the assessment of the overall protein profile of UWMS samples, specifically used to detect carbonic anhydrase VI protein band. Pixel intensity of the carbonic anhydrase band was assessed in order to determine variations at T0, 1 and 2.

Coomassie Brilliant Blue R250 (Sigma) was diluted in acetic acid (according to the manufacturer's instructions). Gels were stained for 30 minutes under agitation and de - stained in 10% (v/v) acetic acid until clear and scanned on an automated image developing system, the ChemiDoc MP imaging system (Bio-Rad, Hemel Hempstead, UK). Chemidoc images were analysed with Image Lab software (Bio-Rad, Hemel Hempstead, UK).

3.3.4 Statistical Analysis

Wilcoxon matched pairs signed rank test was used to indicate differences between same patient concentration and secretion rate at T0 (pre IMRT) in comparison to T1 (6 months post IMRT) and T2 (12 months post IMRT).

Repeated measures Friedman test to assess variation of biochemical parameters, total protein concentration, secretion rate and specific proteins in saliva within the oral cavity of HNC patients at three time point.

Wilcoxon–Mann–Whitney test to indicate the difference between different locations of primary tumour among patients.

Random effects Generalized Least Squares in a longitudinal panel was the model used to analyse the data obtained from several measures taken from the same patients at different times, in order to determine the relationship between two variables making a distinction between independent and dependent variables. Panel refers to a group of subjects that were studied recurrently over time in order to determinate the association between salivary proteins and oral mucositis development at T1 (6 months post IMRT) and T2 (12 months post IMRT) in comparison to T0 (pre IMRT). (Longitudinal and panel data: analysis and applications in the social sciences. EW Frees - 2004).

All analysis was carried out using STATA 15.1 (College Station, Texas USA, www.stata.com), GraphPad Prism 8 software (La Jolla California USA, www.graphpad .com) and Microsoft Excel 2018. Results were expressed as a mean ± SEM relative to the variables and ‘n’ represents the number of subjects. P value was set at <0.05

3.4 RESULTS

3.4.1 Total Protein Concentration (TPC) in UWMS

Mean TPC for patients prior to IMRT (T0) was 2.97 ± 0.31 mg/ml (±SEM). At T1 (6 months post IMRT), TPC was reduced to 2.59 ±0.23 mg/ml, compared with pre-radiotherapy protein concentration. At T2 (12 months post IMRT), the TPC was significantly increased to 3.74 ±0.32 mg/ml in comparison with T0 (p = 0.008) (Figure 3.2).

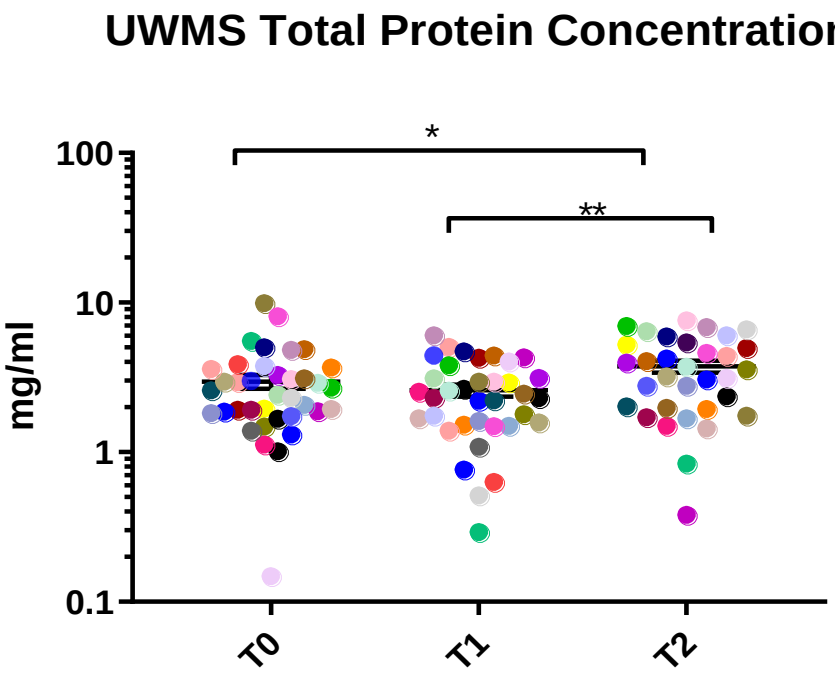


Figure 3. 2 Total protein concentration pre (T0) and post IMRT at 6 months (T1) and 12 months (T2).

TPC changed in UWMS pre and post IMRT. Total protein concentration was lower at T1 compared with T0 *($p = 0.68$)*. Conversely at T 2 was significantly increased compared with T0 and T1 (*$p = 0.049$* and p = *0.008* respectively) (Wilcoxon matched pairs signed rank test). Every colour represents an individual participant. Data is represented as mean ± SEM.

3.4.2 Total Protein Secretion (TPS) rate in UWMS

Mean TPS rate of HNC patients at T0 was 1.22 ± 0.12 mg/min. At T1 the TPS rate was reduced to 0.46 ±0.08 mg/min, which represented a significant reduction compared with pre-radiotherapy (T0) protein secretion rate. At T2, the TPS rate reached 0.76 mg/min (SEM ± 0.10) showing a degree of recovery but remained significantly decreased compared to T0 (Figure 3.3).

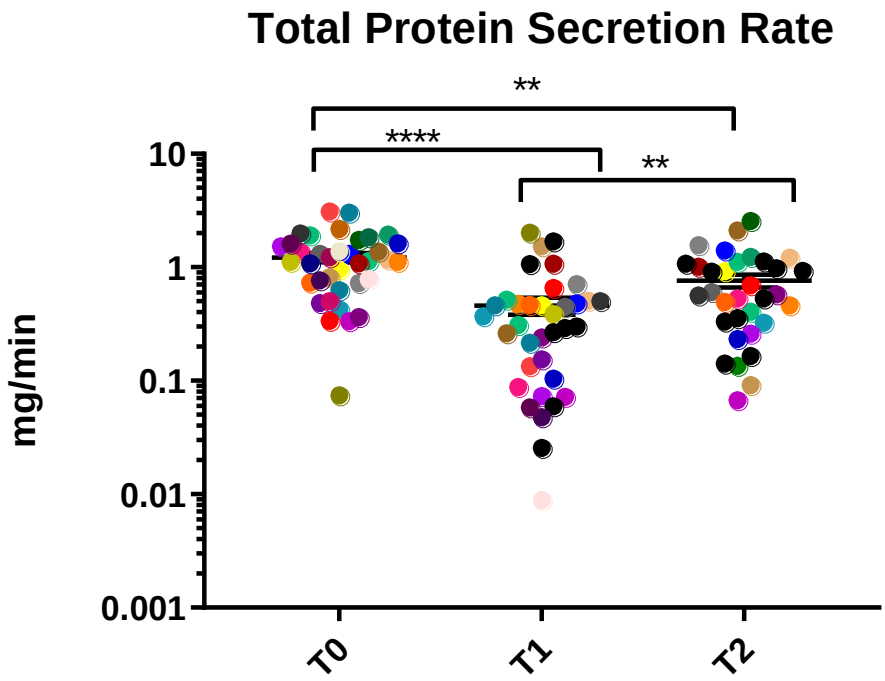


Figure 3.3 10 TPS rate variation pre (T0) and post- IMRT at 6 months (T1) and 12 months (T2).

Total protein secretion rate in UWMS was reduced significantly at the T1 and T2 compared with T0 ($p < 0.0001$ and $p = 0.007$ respectively). TPS rate in UWMS was reduced significantly at T1 in comparison to T2 ($p = 0.003$) (Wilcoxon matched pairs signed rank test) Every colour represents an individual participant. Data is represented as mean ± SEM.

3.4.3 Specific Protein Analysis of UWMS Pre and Post IMRT

3.4.3.1Mucin 5B Concentration in UWMS

Mucin 5B concentration was investigated in UWMS pre-IMRT (T0) and at T1 (6 months post-IMRT) and T2 (12 months post-IMRT). Analysis of mucin 5B concentration post IMRT showed a significant increase at each of the two-time points ($p = 0.0001$) compared with T0 (Friedman Test) (Figure 3.4).

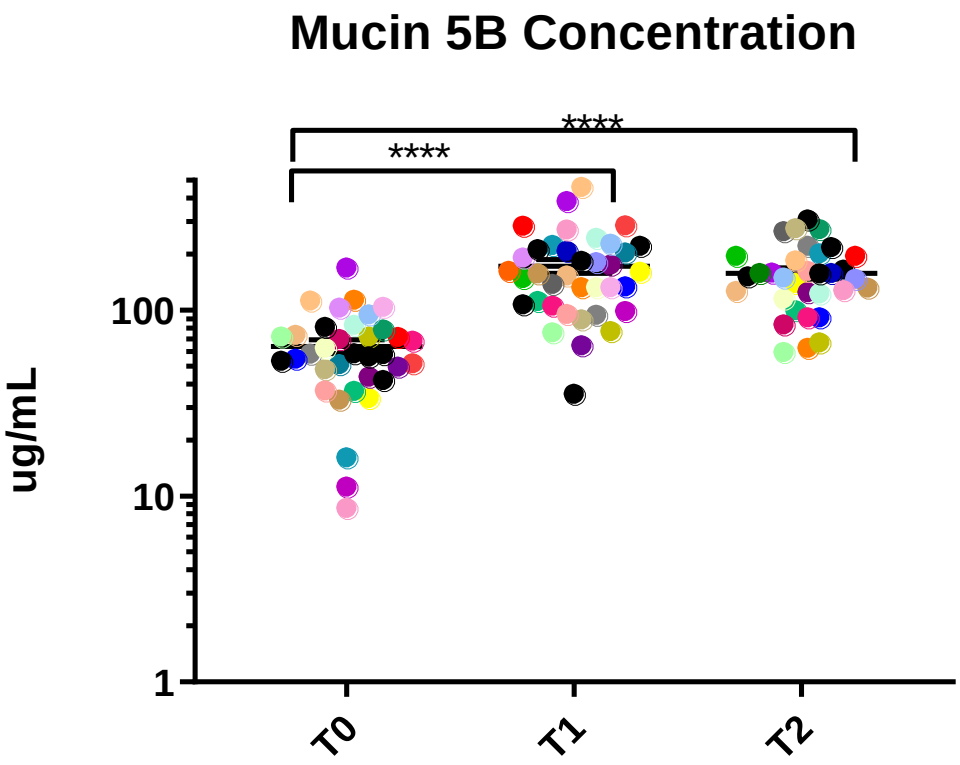


Figure 3. 4 Mucin 5B concentration variation pre- IMRT (T0) and post-IMRT at 6 months (T1) and 12 months (T2).

Mucin 5B concentration in UWMS was increased significantly at T1 and at T2 in comparison to T0 (*p<0.0001*). No statistically significant differences were observed in the mucin 5B concentration at T1 and T2 (*p = 0.269*) (Wilcoxon matched pairs signed rank test) Every colour represents an individual participant. Data is represented as mean ± SEM.

3.4.3.2 Mucin 5B Secretion Rate (Outcome) in UWMS

Mucin 5B secretion rate post-IMRT was slightly increased at T1 (6 months post-IMRT) and T2 (12 months post-IMRT), compared with pre-IMRT (T0), reaching the highest value at T2. However, no significant differences were found (*p = 0.518*) (Figure 3.5).

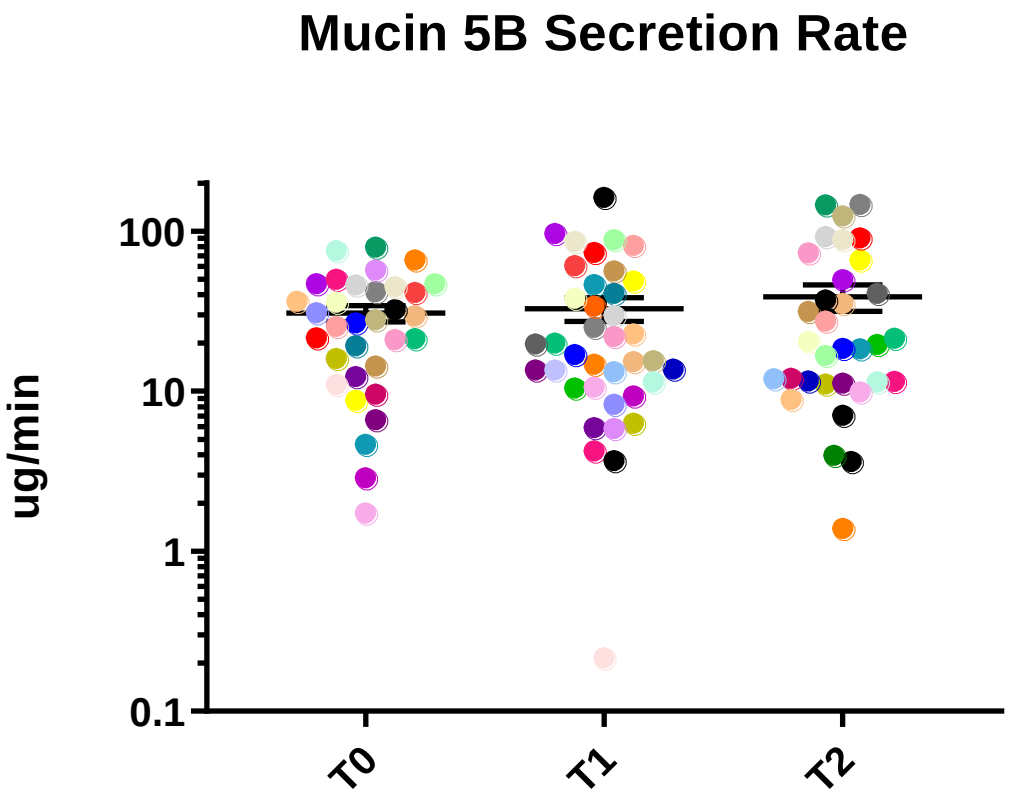


Figure 3.5 11 Mucin 5B secretion rate variation pre-(T0) and post- IMRT at 6 months (T1) and 12 months (T2).

No statistically significant differences were observed in the in mucin 5B secretion rate levels were observed at T1 and T2 compared with time point T0 (*p = 0.458* and *p = 0.2545* respectively) (Wilcoxon matched pairs signed rank test). Every colour represents 1 participant. Data is represented as mean ± SEM.

3.4.3.3 Mucin 7 Concentration in UWMS

Mucin 7 concentration was analysed pre and post IMRT. It was observed that post-IMRT the concentration of mucin 7 increased significantly (p = 0.005) at the two time points (T1 and T2) compared with T0 (Figure 3.6).

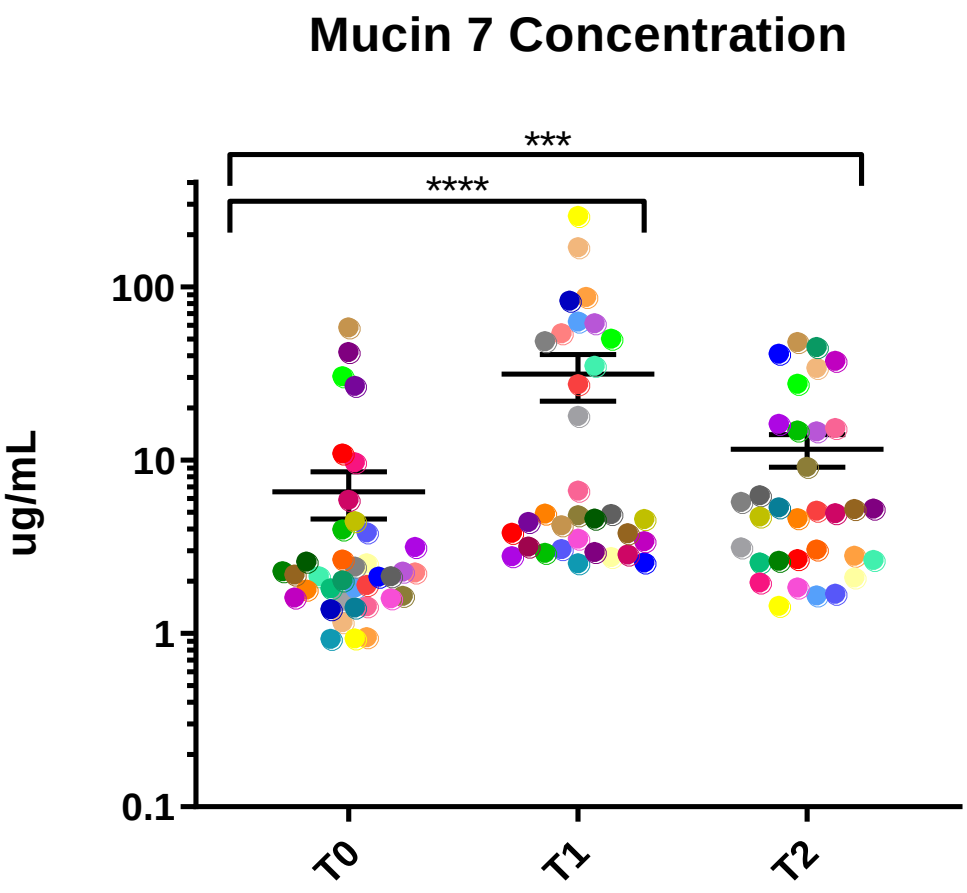


Figure 3.6 Mucin 7 concentration variation pre-(T0) and post IMRT at 6 months (T1) and 12 months (T2).

Mucin 7 concentration in UWMS was increased significantly at T1 and T2 in comparison to T0 (p=0.0001 and p=0.0062 respectively) (Wilcoxon matched pairs signed rank test). Every colour represents 1 participant. Data is represented as mean ± SEM.

3.4.3.4 Mucin 7 Secretion Rate in UWMS

Analysis of mucin 7 secretion rate data post IMRT revealed an increase at T1 (6 months post IMRT) and T2 (12 months post IMRT) compared with T0 (pre-IMRT), reaching the highest value at time T2. However, this increase in secretion rate was not statistically significant ($p = 0.4651$) (Friedman Test) (Figure 3.7).

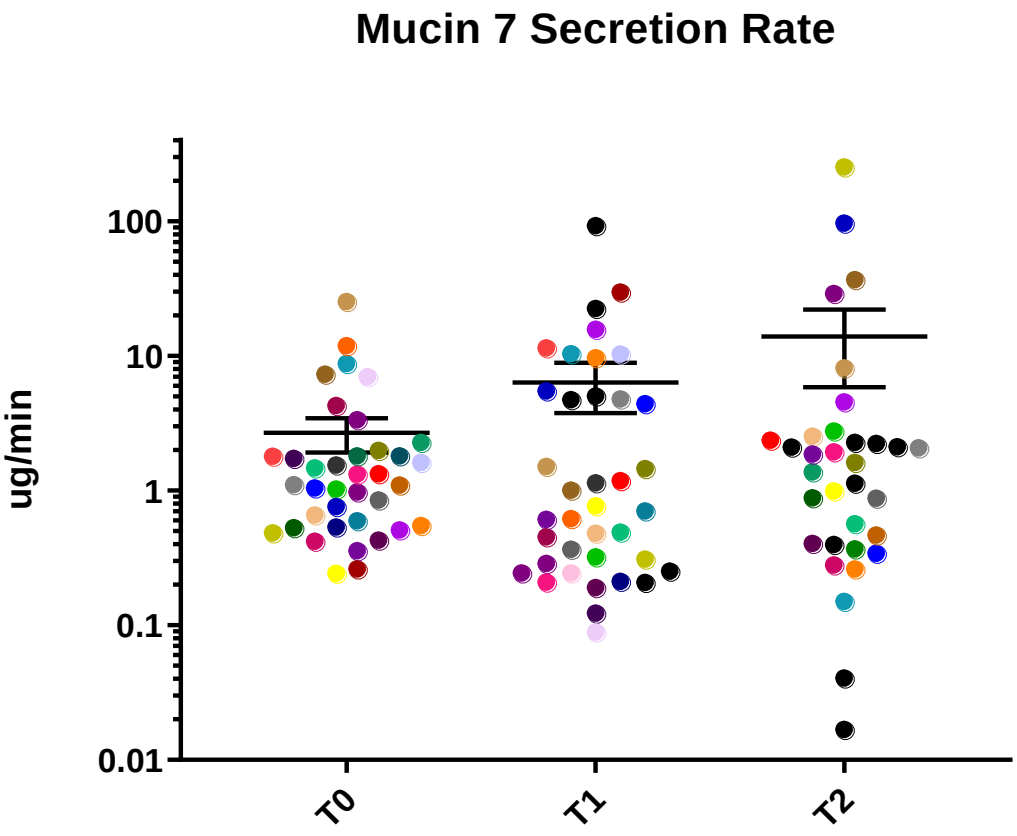


Figure 3. 7 Mucin 7 secretion rate variation pre-(T0) and post IMRT at 6 months (T1) and 12 months (T2).

No significant differences were observed in mucin 7 secretion rate levels between T0 and T1 and T2 (p = 0.6653 and p = 0.3896 respectively). No statistically significant differences were observed in mucin 7 secretion rate between T1 and T2 (p = 0.9779) (Wilcoxon matched pairs signed rank test) every colour represents 1 participant. data is represented as mean ± SEM.

3.4.3.5 Secretory IgA Concentration in UWMS

Analysis of IgA concentration post IMRT showed slight increase at T 1 (6 months post IMRT) and T 2 (12 months post IMRT) compared with T 0 (pre IMRT). IgA concentration reaching the highest value at T 2, however no significant differences were found (p = 0.1699) a cross the three time points (Friedman Test) (Figure 3.8).

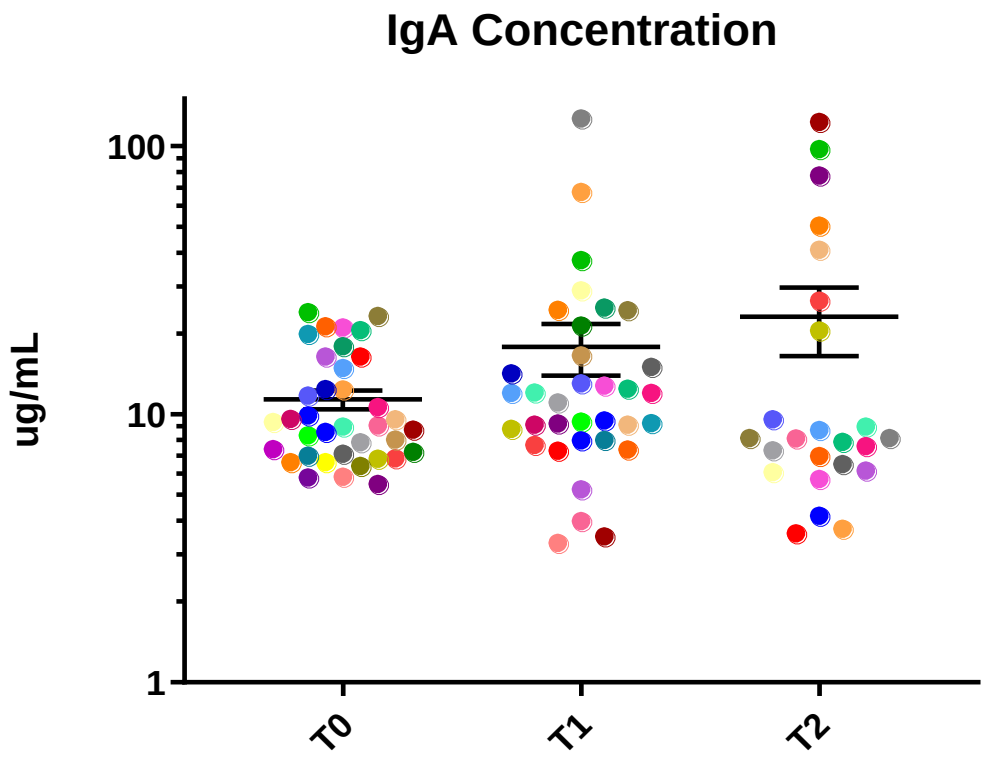


Figure 3.8 IgA concentration variation pre (T0) and post IMRT at 6 months (T1) and 12 months (T2).

No significant difference was observed in IgA concentration at T1 and T2 in comparison to T0 (p = 0.339, p = 0.99 respectively). no significant difference between T1 and T2 (p = 0.8553) (Wilcoxon matched pairs signed rank test). Every colour represents 1 participant. Data is represented as mean ± SEM.

3.4.3.6 IgA Secretion Rate in UWMS

Analysis of IgA secretion rate post IMRT was slightly increased at T1 and T2 compared with T0, reaching the highest value at T2. However, this increase was not statistically significant (p = 0.0841) across the 3 time points (Friedman Test) (Figure 3.9).

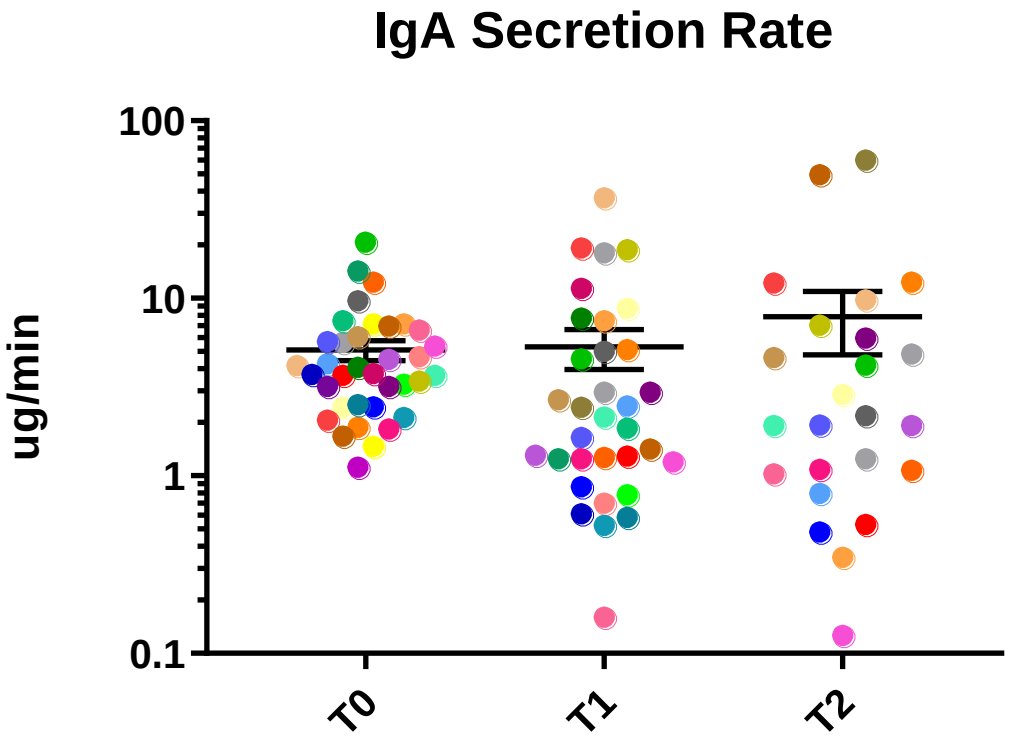


Figure 3.9 IgA secretion rate variation pre (T0) and post IMRT at 6 months (T1) and 12 months (T2).

No significant difference was observed in IgA secretion rate at T1 and T2 in comparison to T0 (p =0.1252 and p= 0.3053 respectively). There was no significant difference between T1and T2 (p =0.5088) (Wilcoxon matched pairs signed rank test). Every colour represents 1 participant. Data is represented as mean ± SEM.

3.4.3.7 α -Amylase Concentration in UWMS

α-Amylase concentration post IMRT was reduced at T1 (55.83 ± 9.57) reaching the lowest level compared with T0 (112.63 ± 16.71) and T2 (111.08 ± 15.40). Though α-amylase concentration variation was not significant (p = 0.073) across the 3 time points (Friedman Test) (Figure 3.10).

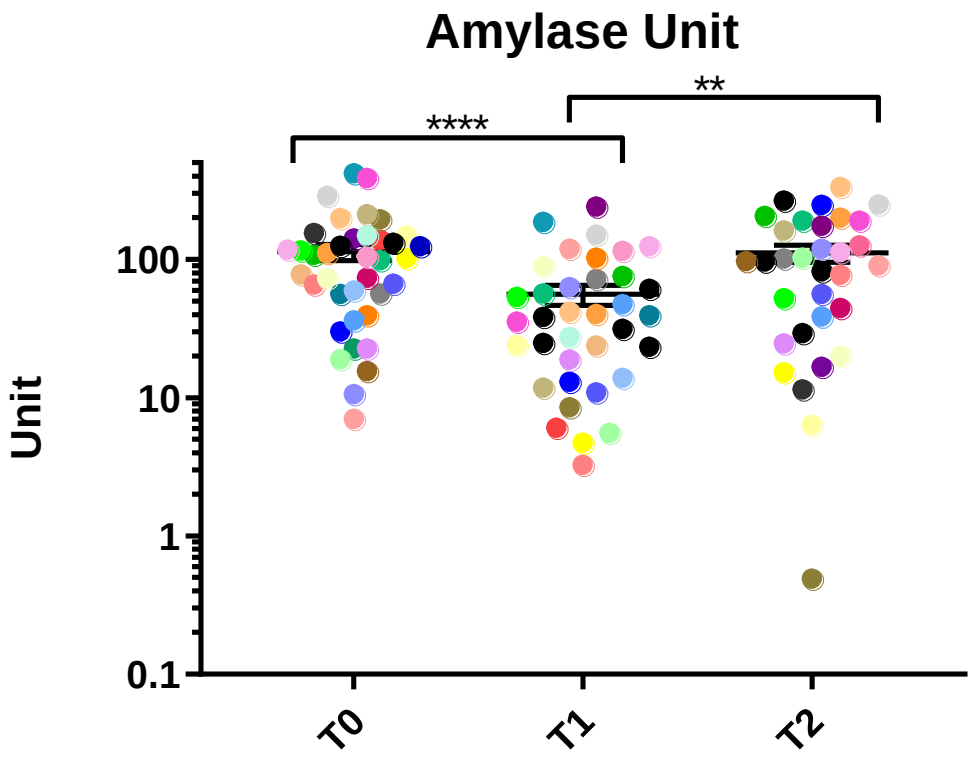


Figure 3.10 α-Amylase unit variation pre (T0) and post IMRT at 6 months (T1) and 12 months (T2).

α-Amylase units in UWMS was reduced significantly at T1 compared with T0 and T2 (p= 0.0003 and p = 0.0081 respectively). No difference was observed between T0 and T2 (p= 0.7) (Wilcoxon matched pairs signed rank test). every colour represents 1 participant. data is represented as mean ± SEM.

3.4.3.8 α-Amylase Unit Secretion Rate in UWMS

α-Amylase unit secretion rate (mean value) post IMRT was reduced at T1 (11.87 ± 3.99) reaching the lowest level compared with T0(48.51 ± 6.77) and T2(23.34 ± 4.34). α-Amylase unit secretion rate variation varied significantly at all 3 time points ($p < 0.0001$) (Friedman Test) (Figure 3.11).

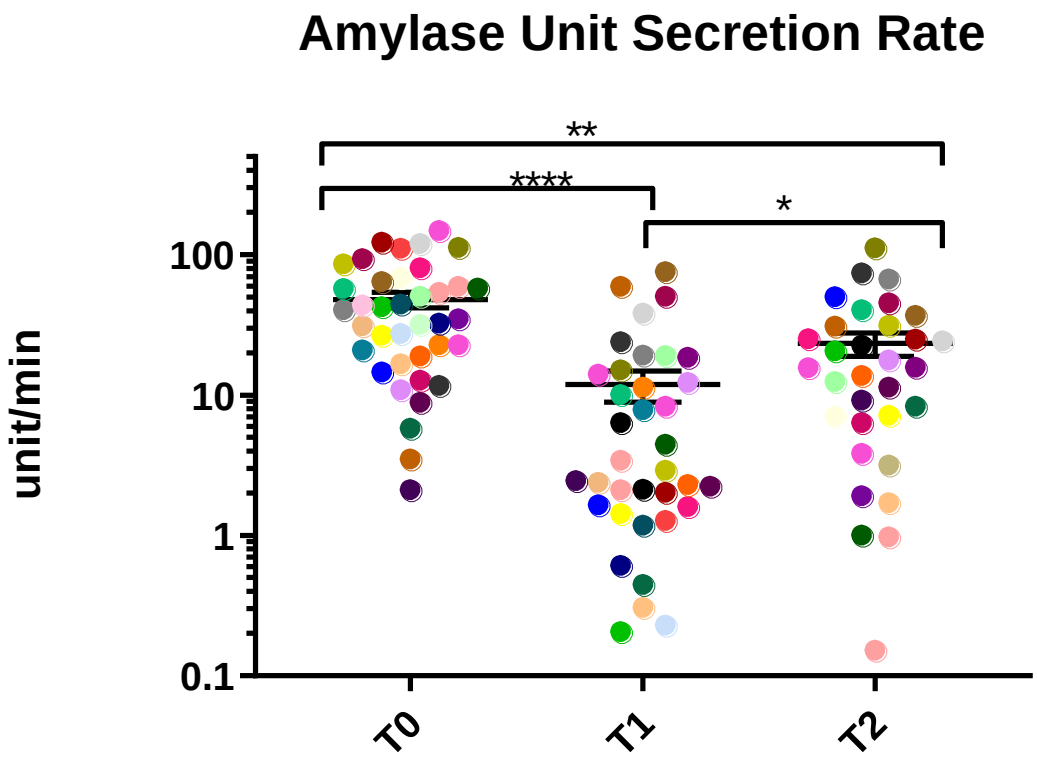


Figure 3.11 α-Amylase secretion rate pre (T0) and post IMRT at 6 months (T1) and 12 months (T2).

α-Amylase unit secretion rate was significantly reduced at T1 and T2 in comparison to T0 ($p < 0.0001$ and p 0.003 respectively). α-amylase unit secretion rate was significantly reduced at T1 in comparison to T2 ($p = 0.0291$) (Wilcoxon matched pairs signed rank test). Every colour represents 1 participant. Data is represented as mean ± SEM.

3.4.3.9 Albumin Concentration in UWMS

Albumin concentration post IMRT was increased at T1 (98.35 ± 13.29) reaching the highest level compared with T0 (64.5 ± 13.01) and T2 (55.8 ± 10.8). Moreover, albumin concentration varied significantly post IMRT (p = 0.005) (Friedman Test) (Figure 3.12).

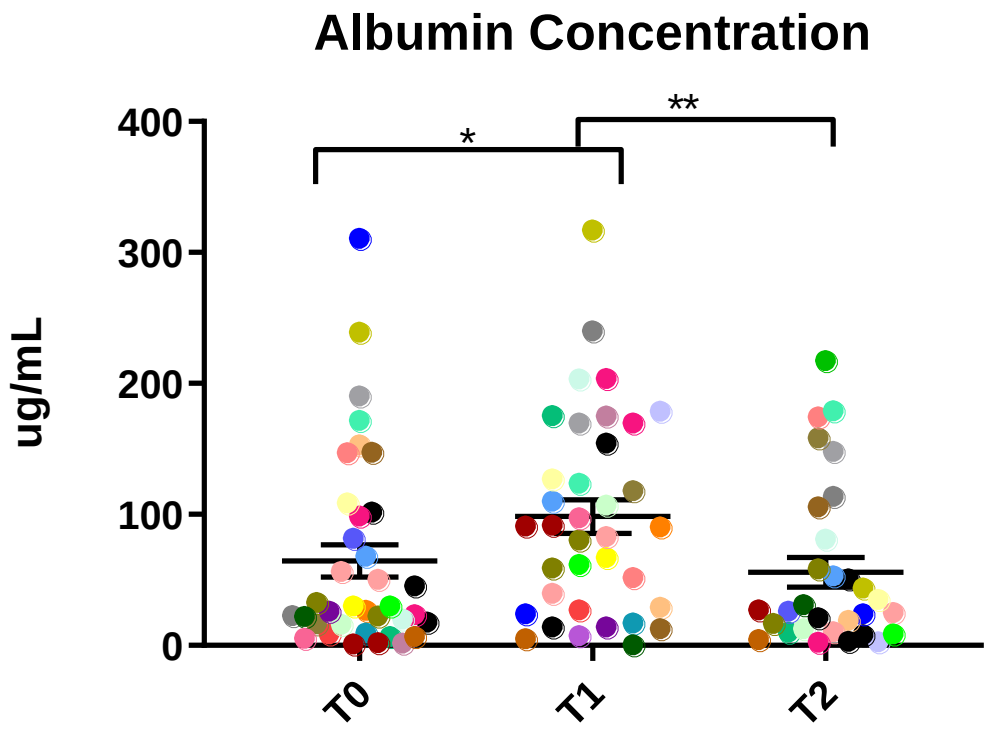


Figure 3.12 Albumin concentration pre (T0) and post IMRT at 6 months (T1) and 12 months (T2).

Albumin concentration in UWMS was significantly increased at T1 in comparison to T0 and T2 (p = 0.0249 and p0.0029 respectively). No statistically significant differences were observed in albumin concentration between T2 and T0 (p = 0.256) (Wilcoxon matched pairs signed rank test). Every colour represents 1 participant. Data is represented as mean ± SEM.

3.4.3.10 Albumin Secretion Rate

Albumin secretion rate post IMRT was decreased at T1 (16.55 ± 3.24) compared with T0 (25.00 ± 5.45) as well as at T2 (11.29 ± 2.76). Moreover, albumin secretion rate varied significantly across the 3 time points (p=0.003) (Friedman Test) (Figure 3.13).

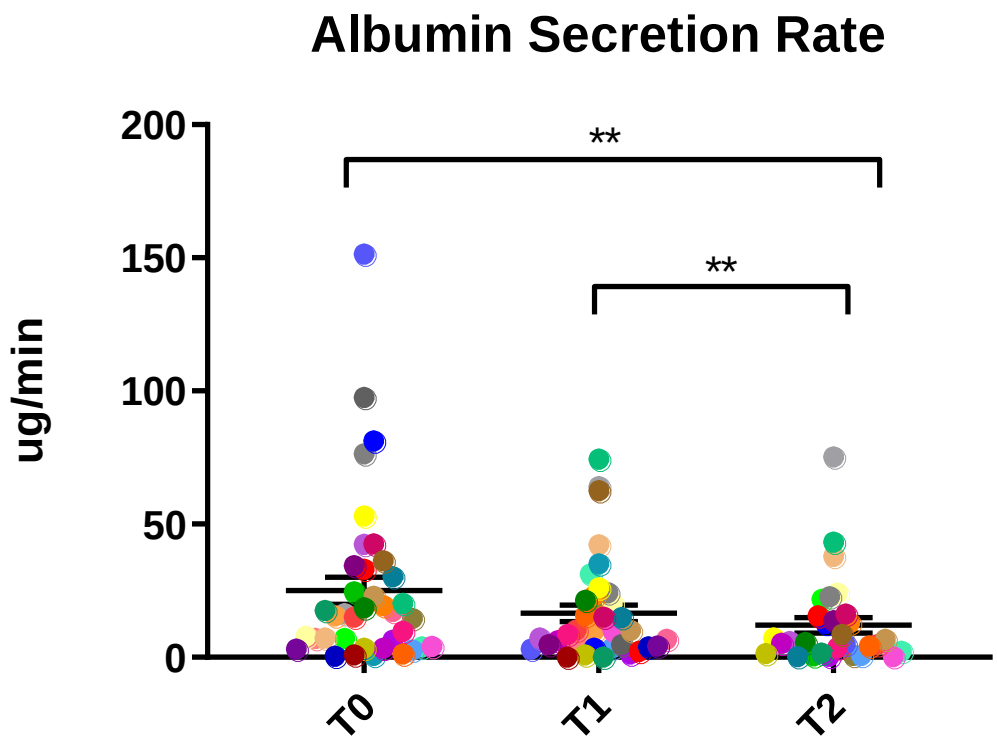


Figure 3.13 Albumin secretion rate pre (T0) and post IMRT at 6 months (T1) and 12 months (T2).

Albumin secretion rate was significantly reduced at T2 in comparison to T0 (p = 0.0057). No significant difference at T1 in comparison to T0 (p=0.3149). Albumin secretion rate was significantly reduced at T2 in comparison to T1 (p = 0.0099) (Wilcoxon matched pairs signed rank test). Every colour represents 1 participant. Data is represented as mean ± SEM.

3.4.3.11 Cystatin S Concentration in UWMS

Cystatin S concentration post IMRT was decreased at T1 (4.68 ±0.57) as well as at T2 (7.50 ±0.99) compared with T0 (14.25 ± 1.039). Cystatin S concentration varied significantly across the 3 time points with $p < 0.001$ (Friedman Test) (Figure 3.14).

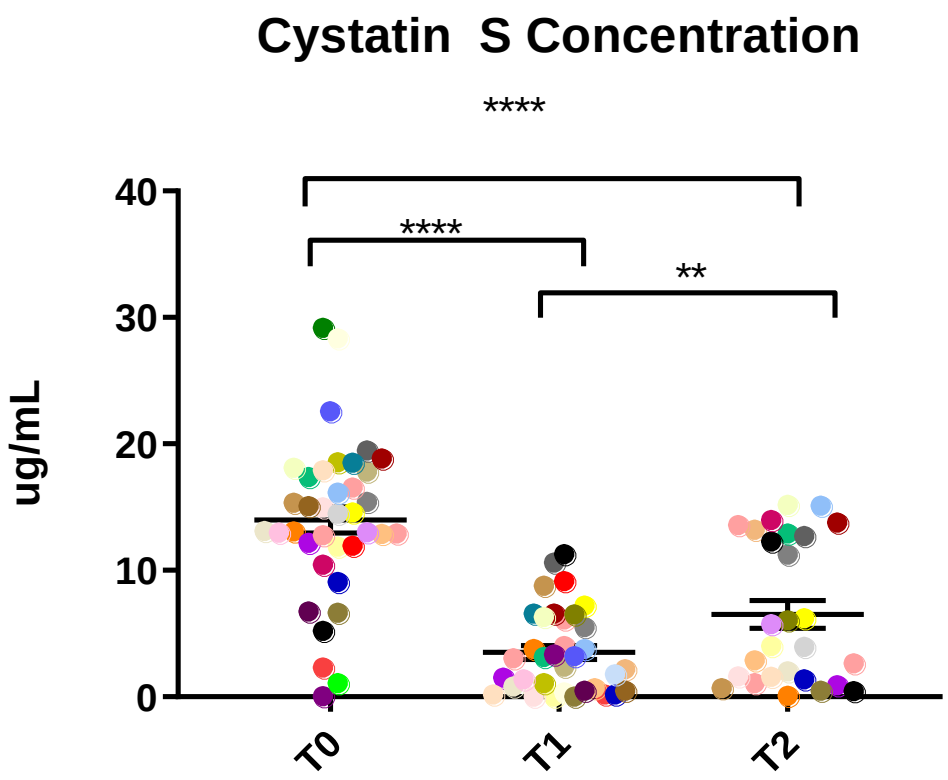


Figure 3.14 Cystatin s concentration variation pre (T0) and post IMRT at 6 months (T1) and 12 months (T2).

Cystatin S concentration in UWMS was significantly decreased at the T1 and T2 in comparison to T0 (p= 0.0001). Cystatin S concentration was significantly decreased at T1 in comparison to T2 (p=0.0096, Wilcoxon matched pairs signed rank test). Every colour represents 1 participant. Data is represented as mean ± SEM.

3.4.3.12 Cystatin S Secretion Rate in UWMS

Cystatin S secretion post IMRT was decrease at T1 (0.88 ±0.24) as well as at T2 (1.99 ± 0.39) compared with T0 (6.98 ± 0.97). Cystatin S concentration varied significantly across the 3 time points p<0.001 (Friedman Test) (Figure 3.15).

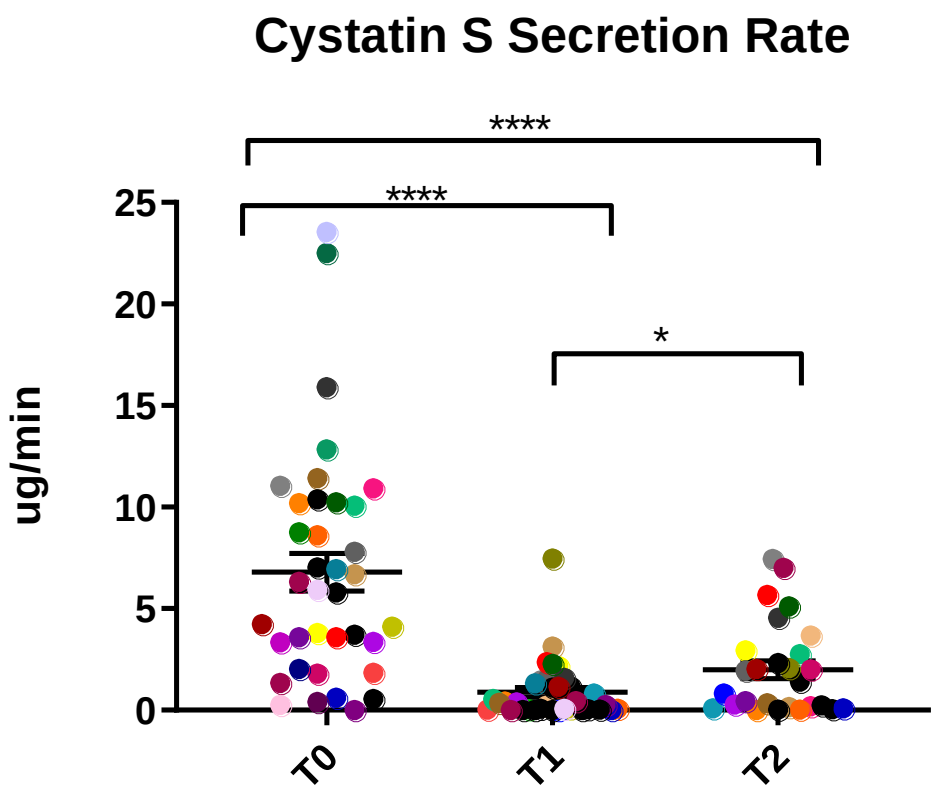


Figure 3.15 Cystatin S secretion rate variation pre (T0) and post IMRT at 6 months (T1) and 12 months (T2).

Cystatin S secretion rate in UWMS was significantly decreased at the T1 and T2 compared with T0 (p<0.0001, p=0.0003 respectively). Cystatin S secretion rate in UWMS was significantly decreased at T1 in comparison to T2 (p=0.0187) (Wilcoxon matched pairs signed rank test). Every colour represents 1 participant. Data is represented as mean ± SEM.

3.4.3.13 Proline Rich Protein (PRP) Concentration in UWMS

PRP concentration post IMRT was slightly decreased at T1 (0.76 ± 0.16) as well as at T2 (0.632 ± 0.11) compared with T0 (0.889 ± 0.20). PRP concentration in UWMS did not vary across the 3 time points (p = 0.8973) (Friedman Test) (Figure 3.16).

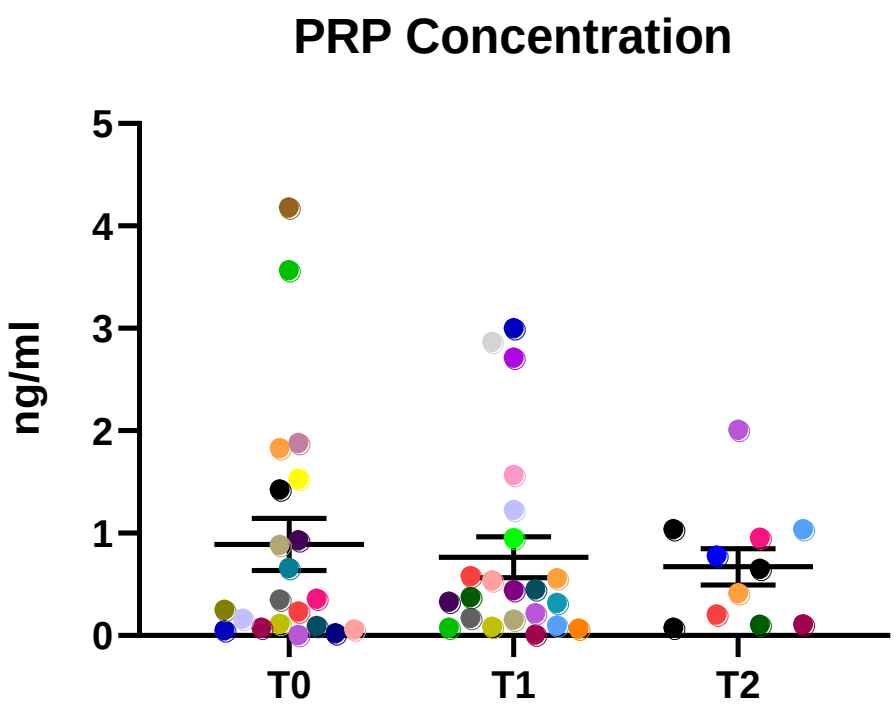


Figure 3.16 a Proline rich protein (PRP) concentration variation pre (T0) and post IMRT at 6 months (T1) and 12 months (T2).

No significant differences in PRP concentration at T1 and T2 compared with T0 (Wilcoxon matched pairs signed rank test). Every colour represents 1 participant. Data is represented as mean ± SEM.

3.4.3.14 Statherin Concentration in UWMS

Statherin concentration post IMRT was decreased at T1 (3.29 ±0.4) compared with T0 (4.55 ± 0.48). In contrast at T2 this was increased reaching the highest values (7.57 ± 1.10). There was no significant difference between statherin concentration across the 3 time points (p = 0.72) (Friedman Test) (Figure 3.17).

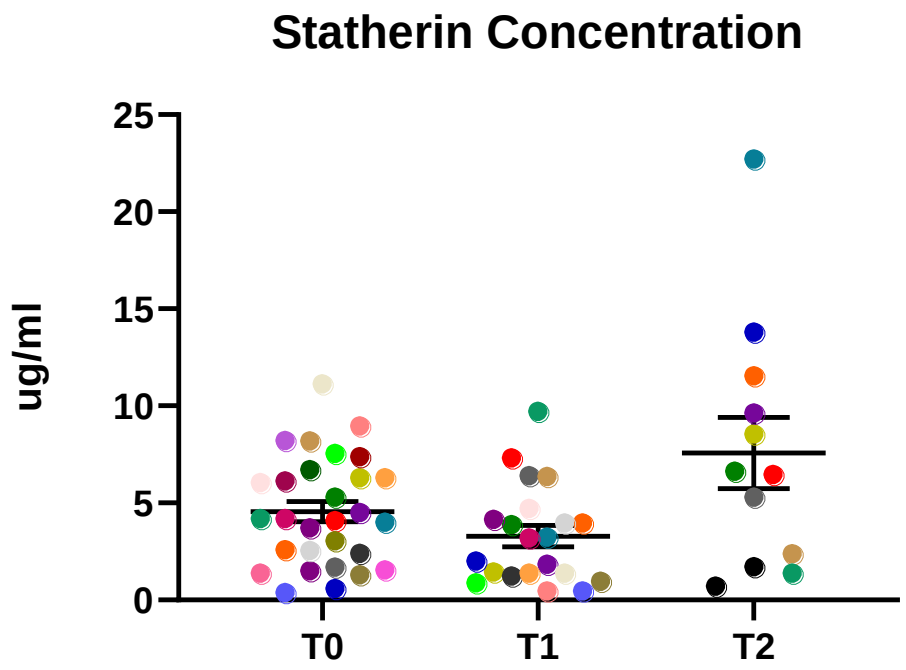


Figure 3.17 Statherin concentration variation pre (T0) and post IMRT at 6 months (T1) and 12 months (T2).

No significant difference in Statherin concentration seen at T1 and T2 compared with T0 (p = 0.6982 and p = 0.1763 respectively). No difference was observed between T1 and T2 concentration (p 0.3804) (Wilcoxon matched pairs signed rank test). Every colour represents 1 participant. Data is represented as mean ± SEM.

3.4.3.15 Carbonic Anhydrase VI (CA VI) Pixel Intensity

Post IMRT CA VI pixel intensity (mean value) showed an increase at T1 (2413 ± 418.2) as well as at T2 (1596 ± 227.44) in comparison with T0 (1464.97 ± 27). No significant differences in CA VI pixel intensity were observed post IMRT (p = 0.9285) (Friedman Test) (Figure 3.27).

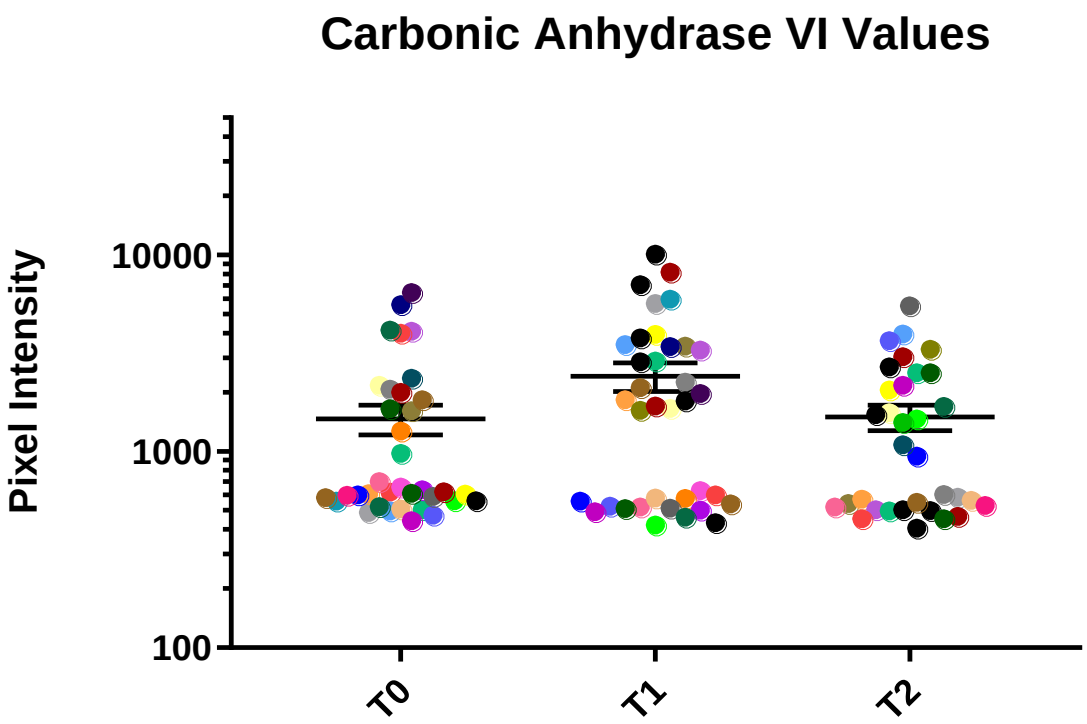


Figure 3.18 Carbonic Anhydrase VI (CAVI) pixel intensity in UWMS variation pre (T0) and post IMRT at 6 months (T1) and 12 months (T2).

CA VI pixels intensity in UWMS was increased significantly at T1compared with T0 (p0.0498). No significant difference at T2 in comparison to T0 pixels intensity (p = 0.893) (Wilcoxon matched pairs signed rank test). Every colour represents 1 participant. Data is represented as mean ± SEM.

3.4.3.16 Carbonic Anhydrase VI (CA VI) Pixel Intensity Secretion Rate

Post IMRT CA VI pixel intensity secretion rate decreased at T1 (427.1 ± 92.51) as well as at T2 (269.5 ± 51.66) compared with T0 (668.2 ±132.3), reaching the lowest point at T2. Significant differences in CA VI pixel intensity secretion rate were observed at T1 and T2 compared to T0 (p=0.0065) (Figure 3.19).

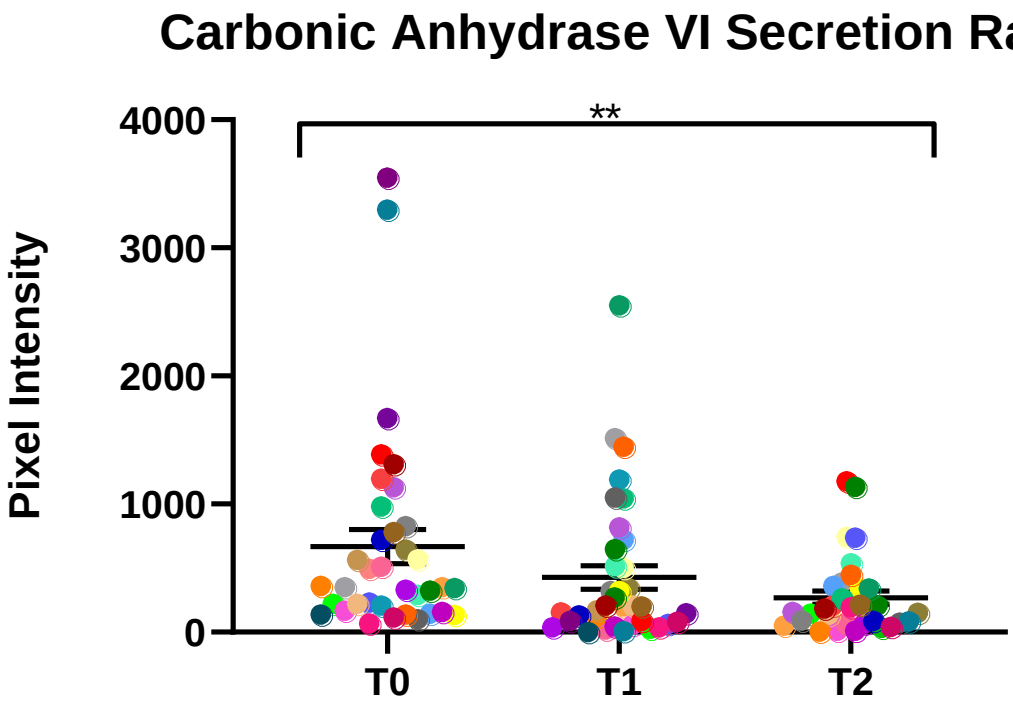


Figure 3.19 Carbonic Anhydrase VI (CA VI) Coomassie blue pixel intensity in UWMS variation pre (T0) and post IMRT at 6 months (T1) and 12 months (T2).

CA VI pixels intensity secretion rate in UWMS was decreased significantly at the T2 compared with T0 (p 0.0243) (Wilcoxon matched pairs signed rank test). Every colour represents 1 participant. Data is represented as mean ± SEM.

3.4.4 Longitudinal associations between clinical assessment outcomes and salivary protein analysis post IMRT at 6 months (T1) and 12 months (T2) by using Random effects Generalised Least Squares linear regression.

In addition to the analysis of the influence of IMRT on these salivary proteins, it was assessed the possible association between clinical measures reported in Chapter 2 and total protein concentration, secretion rate along with these specific salivary proteins regarding their functions that represent clinical relevance in maintaining salivary properties. Protein variation in concentration and secretion rate might hasten the onset and progression of oral diseases along with changing saliva viscoelastic and protective properties (Hannig et al., 2017; Pitts et al., 2017).

Salivary flow rate

In this section it was investigated the probable association between altered biochemical composition of saliva and the significantly reduced salivary flow rate post IMRT that was reported in chapter 2, in order to analyse the dependence between clinical observations and biological composition of saliva that make up its functionality (Jawad et al., 2015).

When comparing associations between oral salivary flow rate and total protein concentration and secretion rate there was a significant association at both time points post IMRT (Table 3.1 and 3.2)

Regarding mucins 5B and 7, there were statistically significant association between increased concentrations of both mucins in a reduced salivary volume. Mucins secretion rate was not significantly different after IMRT (Figure 3.5 and 3.7), and significantly associated with salivary flow rate post IMRT (Table 3.4 and 3.6)

This study found that there were statistically significant associations for α-amylase unit and secretion rate and salivary flow rate at T1 and T2 ($p < 0.0001$) (Table 3.7 and 3.8). In keeping with the pattern of significance at T1 and T2, a reduced cystatin S concentration and secretion rate (Figure3.14 and 3.15) were both significantly associated with salivary flow rate at Time Point 1 and 2 (Table 3.9 and 3.10)

Salivary flow rate

Table 3.1 Association between salivary flow rate and total protein concentration (TPC) post-IMRT at 6 months (T1) and 12 months (T2).

SFR / TPC	Coefficient	P	[95% Conf.	Interval]
T0	-0.025	0.004	-0.043	-0.008
T1	-0.308	**0.0001**	-0.373	-0.242
T2	-0.197	**0.0001**	-0.265	-0.129

Table shows significant association between salivary flow rate and total protein concentration (TPC) post-IMRT at 6 months(T1) (p=0.0001) and significant correlation at 12 months (T2) (p=0.001).

Table 3.2 Association between salivary flow rate and total protein secretion rate (TP SR) post-IMRT at 6 months (T1) and 12 months (T2).

SFR / TP SR	Coefficient	P	[95% Conf.	Interval]
T0	0.146	0.0001	0.099	0.193
T1	-0.185	**0.0001**	-0.255	-0.115
T2	-0.146	**0.0001**	-0.211	-0.080

Table shows significant association between salivary flow rate (SFR) and total protein secretion rate (TP SR) post-IMRT at 6 months (T1) (p=0.0001) and significant correlation at 12 months (T2) (p=0.001).

Table 3.3 Association between salivary flow rate and mucin 5B (MUC 5B) concentration post-IMRT at 6 months (T1) and 12 months (T2).

SFR / MUC 5B	Coefficient	P	[95% Conf.	Interval]
T0	0 .0003	0.157	-0.0001	0.0008
T1	-0.341	**0.0001**	-0.426	-0.255
T2	-0.257	**0.0001**	-0.341	-0.173

Table shows significant association between salivary flow rate (SFR) and MUC 5B concentration post-IMRT at 6 months (T1) (p=0.0001) and significant correlation at 12 months (T2) (p=0.001).

Table 3.4 Association between salivary flow rate and mucin 5B secretion rate (MUC 5B SR) post-IMRT at 6 months (T1) and 12 months (T2).

SFR /MUC 5B SR	Coefficient	P	[95% Conf.	Interval]
T0	0.004	0.0001	0.0033	0.004
T1	-0.315	**0.0001**	-0.373	-0.257
T2	-0.258	**0.0001**	-.0319	-0.198

Table shows significant association between salivary flow rate (SFR) and MUC 5B SR post-IMRT at 6 months (T1) (p=0.0001) and significant correlation at 12 months (T2) (p=0.001).

Table 3.5 Association between salivary flow rate and mucin 7 (MUC 7) concentration post-IMRT at 6 months (T1) and 12 months (T2).

SFR/ MUC 7	Coefficient	P	[95% Conf.	Interval]
T0	0.0002	0.634	-0.0007	0.001
T1	-0.299	**0.0001**	-0.366	-0.231
T2	-0.216	**0.0001**	-0.283	-0.148

Table shows significant association between salivary flow rate (SFR) and MUC 7 concentration post-IMRT at 6 months (T1) (p=0.0001) and significant correlation at 12 months (T2) (p=0.001).

Table 3.6 Association between salivary flow rate and mucin 7 secretion rate (MUC 7 SR) post-IMRT at 6 months (T1) and 12 months (T2).

SFR/ MUC7 SR	Coefficient	P	[95% Conf.	Interval]
T0	0.0002	0.674	-0.0008	0.001
T1	-0.294	**0.0001**	-0.359	-0.230
T 2	-0.217	**0.0001**	-0.285	-0.149

Table shows significant association between salivary flow rate (SFR) and MUC 7 secretion rate post-IMRT at 6 months (T1) (p=0.0001) and significant correlation at 12 months (T2) (p=0.001).

Table 3.7 Association between salivary flow rate and α-amylase unit (U) post-IMRT at 6 months (T1) and 12 months (T2).

SFR /α-AMYLASE U	Coefficient	P	[95% Conf.	Interval]
T0	0.0004	0.046	-0.0008	-7.71e-06
T1	-0.323	**0.0001**	-0.395	-0.251
T2	-0.214	**0.0001**	-0.285	-0.143

Table shows significant association between salivary flow rate (SFR) and α-amylase U post-IMRT at 6 months (T1) (p=0.0001) and significant correlation at 12 months (T2) (p=0.001).

Table 3.8 Association between salivary flow rate and α-amylase unit secretion rate (U SR) post-IMRT at 6 months (T1) and 12 months (T2).

SFR/ α-AMYLASE U SR	Coefficient	P	[95% Conf.	Interval]
T0	0.002	0.0001	0.001	0.003
T1	-0.200	**0.0001**	-0.276	-0.125
T 2	-0.145	**0.0001**	-0.218	-0.072

Table shows significant association between salivary flow rate (SFR) and α-amylase U SR post-IMRT at 6 months (T1) (p=0.0001) and significant correlation at 12 months (T2) (p=0.001).

Table 3.9 association between salivary flow rate and cystatin S concentration post-IMRT at 6 months (T1) and 12 months (T2).

SFR/ Cystatin S	Coefficient	P	[95% Conf.	Interval]
T0	0.011	0.001	0.004	0.018
T1	-0.196	**0.0001**	-0.290	-0.102
T 2	-0.143	**0.001**	-0.229	-0.058

Table shows significant association between salivary flow rate (SFR) and cystatin S concentration post-IMRT at 6 months (T1) (p=0.0001) and significant correlation at 12 months (T2) (p=0.001).

Table 3.10 Association between salivary flow rate and cystatin S secretion rate (SR) post-IMRT at 6 months (T1) and 12 months (T2).

SFR / Cystatin S SR	Coefficient	P	[95% Conf.	Interval]
T0	0.028	0.0001	0.021	0.035
T1	-0.141	**0.0001**	-0.210	-0.071
T2	-0.076	**0.034**	-0.146	-0.005

Table shows significant association between salivary flow rate (SFR) and cystatin S SR post-IMRT at 6 months (T1) (p=0.0001) and significant correlation at 12 months (T2) (p=0.034).

Dry mouth report

Dry mouth feeling reported by patients through answering a question extracted from a full questionnaire (Fox et al., 1987) was significantly increased a post IMRT at time point 1 and 2.

Total protein secretion rate was significantly reduced post IMRT (Figure 3. 3) and positively and significantly associated with dry mouth feeling reported by patients after IMRT at time point 1 and 2 (Table 3. 11). Additionally, dry mouth feeling reported by patients showed a strong positive association with mucin 5B and 7 concentration (Table 3.12 and 3.14) and secretion rate (Table 3.13 and 3.15); there were both significantly associated at T1 and T2.

Table 3.11 Aassociation between dry mouth feeling reported by patients and total protein secretion rate (TP SR) post-IMRT at 6 months (T1) and 12 months (T2).

Dry mouth feeling/TP SR	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	-0.035	0.302	-0.101	0.031
T1	0.890	**0.0001**	0.789	0.991
T2	0.920	**0.0001**	0.825	1.015

Table shows significant association between dry mouth feeling reported by patients and TP SR post-IMRT at 6 months(T1) (p=0.0001) and significant correlation at 12 months (T2) (p=0.0001).

Table 3.12 Association between dry mouth feeling reported by patients and mucin 5B concentration (MUC 5B) post-IMRT at 6 months (T1) and 12 months (T2).

Dry mouth feeling/ MUC 5B	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	-0.0002	0.349	-0.0009	0.0003
T1	0.949	**0.0001**	0.835	1.062
T 2	0.962	**0.0001**	0.851	1.073

Table shows significant association between dry mouth feeling reported by patients and mucin 5B post-IMRT at 6 months (T1) (p=0.0001) and significant correlation at 12 months (T2) (p=0.0001).

Table 3.13 Association between dry mouth feeling reported by patients and mucin 5B secretion rate (MUC 5B SR) post-IMRT at 6 months (T1) and 12 months (T2).

Dry mouth feeling/MUC5BSR	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	-0.002	0.1	-0.003	-0.001
T1	0.925	**0.0001**	0.836	1.013
T2	0.956	**0.0001**	0.864	1.048

Table shows significant association between dry mouth feeling reported by patients and MUC 5B SR post-IMRT at 6 months(T1) (p=0.0001) and significant correlation at 12 months (T2) (p=0.0001).

Table 3.14 Association between dry mouth feeling reported by patients and mucin 7 concentration (MUC 7) post-IMRT at 6 months (T1) and 12 months (T2).

Dry mouth feeling/ MUC 7	Coefficient	P	[95% Conf.	Interval]
T0	0.00005	0.933	-0.0011	0.001
T1	0.917	**0.0001**	0.828	1.007
T 2	0.936	**0.0001**	0.847	1.024

Table shows significant association between dry mouth feeling reported by patients and mucin 7 post-IMRT at 6 months(T1) (p=0.0001) and significant correlation at 12 months (T2) (p=0.0001).

Table 3.15 Association between dry mouth feeling reported by patients and mucin 7 secretion rate (MUC 7 SR) post-IMRT at 6 months (T1) and 12 months (T2).

Dry mouth feeling/ MUC7 SR	Coefficient	P	[95% Conf.	Interval]
T0	0.0001	0.821	-0.001	0.001
T1	0.918	**0.0001**	0.832	1.003
T 2	0.934	**0.0001**	0.844	1.024

Table shows significant association between dry mouth feeling reported by patients and mucin 7 SR post-IMRT at 6 months(T1) (p=0.0001) and significant correlation at 12 months (T2) (p=0.0001).

Taste report

Taste variation reported by patients through answering a question extracted from a full questionnaire (LENT-SOMA) was significantly increased a post IMRT at time point 1 and 2.

CA VI concentration post IMRT (Figure 3.18) was negatively and significantly associated with an increased taste variation reported by patients at T1 (*p=0.0001.)* and T2 (*p=0.003),* (Table 3.16) in the same pattern a reduced CAVI secretion rate which appeared to be significantly associated at both time point (Table 3.17).

Table 3.16 Association between presence of taste alteration dysgeusia reported by patients and carbonic anhydrase VI (CA VI) concentration post-IMRT at 6 months (T1) and 12 months (T2).

Taste report/CAVI	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	-0.0001	0.576	-0.0003	0.0005
T1	-0.614	**0.0001**	-0.0801	-0.428
T2	-0.501	**0.0001**	-0.700	-0.303

Table shows significant association between taste variation reported by patients and CA VI concentration post-IMRT at 6 months(T1) (p=0.0001) and significant correlation at 12 months (T2) (p=0.003).

Table 3.17 Association between presence of taste alteration dysgeusia reported by patients and carbonic anhydrase VI (CA VI) secretion rate (SR) post-IMRT at 6 months (T1) and 12 months (T2).

Taste report/ CAVI SR	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	-0.0001	0.077	-0.002	0.0001
T1	-0.676	0.0001	-0.853	-0.5
T2	-0.465	0.0001	-0.661	-0.269

Table shows significant association between taste variation reported by patients and CA VI SR post-IMRT at 6 months(T1) (p=0.0001) and significant correlation at 12 months (T2) (p=0.0001).

Table 3.18 Summary of protein variation post IMRT at T1 and T2.

UWMS	T1	T2
Total protein concentration	<NS	>*
Total protein secretion rate	<****	<**
MUC 5B concentration	>****	>****
MUC 5B secretion rate	> NS	>NS
MUC 7 concentration	>****	>***
MUC 7 secretion rate	>NS	>NS
IgA concentration	>NS	>NS
IgA secretion rate	>	>
α-Amylase U	<****	>NS
α-Amylase secretion rate	<****	<**
Albumin concentration	>*	<NS
Albumin secretion rate	>NS	>**
Cystatin S concentration	<****	<****
Cystatin S secretion rate	<****	<****
PRP concentration	<NS	<NS
Statherin concentration	<NS	>NS
CA VI Pixel intensity	>NS	NS
CAVI Pixel secretion rate	<NS	<**

Table shows variation of protein concentration and secretion rate variation compared with baseline T0.

3.5 Discussion

It is important to note that there is a small number of longitudinal studies that observed the biochemical composition of saliva in HNC patients undergoing radiotherapy. The vast majority of studies in HNC patients that evaluated salivary proteins were performed during and /or post radiotherapy (short term follow up), collecting different types of saliva (stimulated, UWMS, gland separately), analysing a limited number of biochemical components and providing contradictory results. They attempted to draw conclusions regarding radiotherapy oral sequelae linked with quality of life, leaving more questions than answers. Additionally, many of the studies were cross-sectional and collected saliva post radiotherapy only, without baseline data collected prior to radiotherapy (Funegård et al., 1994; Almståhl et al., 2001; Eliasson et al., 2005; Hannig et al., 2006; Vidotto et al., 2010; Dijkema et al., 2012; Laheij et al., 2015).

It is presumed that such study limitations were due to the challenges of sample collection in this group of patients, due to the lack of a steady salivary flow in HNC patients post-conventional radiotherapy (Almståhl et al., 2001), along with the development of side effects during and post cancer therapy. The presenting oral symptoms and sequelae can make saliva sample collection difficult. In addition, cancer patients will have multiple medical and dental assessments pre and post radiotherapy, reducing study protocol compliance.

Another reason for the limited scope of past studies, it is the difficulty in reproducing standardised conditions during saliva collection techniques. For example, it is crucial to maintain similar clinical conditions (drinking, eating, time of the day) to avoid circadian variations in salivary flow rate and composition, whilst also maintaining standardised methods of transport, storage and laboratory testing to assess salivary components.

Another important factor is clinical staff availability and additional time needed during their dental appointment to collect samples. Moreover, usually studies analysing salivary composition are expressed in the concentration of proteins per millilitres of saliva present in the oral cavity only, without considering secretion rate of proteins which is a stronger indicator, due to the consideration of salivary flow rate.

In this context of the limited scope of past studies and to the author's knowledge, the present study is one of the largest longitudinal studies in HNC patients analysing the organic composition and secretion rate of unstimulated whole mouth saliva pre and post IMRT, enrolling 40 patients, across a period of 12 months following IMRT, providing a comprehensive analysis of salivary component variation post IMRT, and establishing a baseline prior to radiotherapy. The 9 proteins that were selected for analysis are all associated directly with oral health, mucosal and teeth protection, antibacterial, rheological properties, remineralisation microbial homeostasis; therefore they can have the potential to act as predictive markers of the two most common acute and late radiotherapy side effects - oral mucositis and radiotherapy caries, as well as other oral symptoms such as dry mouth and taste perception.

Given that past studies of IMRT have found that it does not fully spare the surrounding tissues, when measured in terms of salivary flow rate (Dijkema et al., 2008), xerostomia (Dijkema et al., 2010), and other oral symptoms such as hyposalivation in general (Nutting et al., 2011; Nguyen et al., 2018), this study proposed the hypothesis that ionising-radiation, delivering a high radiation dose to the tumour, with PG-sparing IMRT, has no sparing effects on the surrounding tissues of salivary glands, and will still affect the salivary output, specific protein concentrations and secretion rate profile at 6- and 12-months post -IMRT.

Past studies have established that altering protein compositions will increase the possibility of disturbing the equilibrium in microbial species in the oral cavity, reducing remineralisation, increasing erosion, and reducing food clearance process resulting in higher risk of developing oral diseases (Carpenter, 2013a; H. L. Gibbins et al., 2014; Kilian et al., 2016; Hannig et al., 2017). In order to measure these factors, UWMS, total protein concentration and secretion rate were measured at every time point as a general indicator of altered salivary gland function post IMRT.

3.5.1 Total protein Concentration and Secretion Rate

Total protein concentration, at T1, was reduced (Figure 3.2) however not to a statistically significant extent. Furthermore, the reduction was not to the same extent as the observed reduction in total protein secretion rate (Figure 3.3).

However, whilst total protein concentration recovered, and even increased to a statistically significant extent beyond baseline figures, at T2 (12 months post-IMRT), in contrast, the total protein secretion rate remained significantly reduced in comparison to baseline T0. The increase in concentration is explained by the significant reduction in the volume of saliva along with the rose in secretion rate at T2 compared with T1.
In addition, Table 3.1 showed negative and significant correlations between these two parameters post IMRT at both time points. A lower secretion rate is indicative of decreased protein production and could be explained by the direct toxicity from radiotherapy in salivary glands during cancer treatment, producing direct DNA damage, impairing the cellular division leading to death and senescence of those cells that were trying to complete this process, as a result acinar cells will not be replenished with new ones, along with general inflammatory reaction and fibrosis of the gland interstitium, (Vissink et al., 2010; Pringle et al., 2013), these might be the causations that result in salivary gland hypofunction clinically observed as a reduced flow rate, altered composition and symptoms that patients reported. However, this association between cell apoptosis and salivary gland altered function remains unclear in the literature. Another possible explanation, suggested by past studies is cell membrane damage induced by RT leading to lysis of acinar cells and granules leakage will induce salivary gland dysfunction (Vissink et al., 2010; Pringle et al., 2013).

This study reported that salivary gland dysfunction, when observed in the form of salivary flow rate and total protein secretion rate, continues one-year post IMRT. Furthermore, the study demonstrated significant associations post IMRT at T1 and T2 (Table 3.2) between these two parameters.

These outcomes are in keeping with findings from past studies that have shown biochemical compositional changes, as well as flow rates, are altered post radiotherapy – whether the radiotherapy was conventional radiotherapy or IMRT. In general, patients that have received

more than 30 Gy are affected by such changes (Dijkema et al., 2012; Gao et al., 2016; Richards et al., 2017).

Conversely, the study conducted by Hannig et al.,(2006), analysing UWMS from 10 HNC patients post radiotherapy compared to 10 control subjects, reported no differences in total protein concentration. However, a high variation among this small group of cancer patients was found. The exact number of months post radiotherapy of saliva collection for each patient was not discussed, which is important as has been shown in this study, total protein concentrations are significantly different between 6- and 12-months post IMRT.

Therefore, in order to further solidify the theory, given Hanning et al 2006 data's, further analysis was carried out to identify the specific protein concentration and secretion rate alterations associated with specific glands. This would allow identification of the clinical avenues to handle IMRT follow-ups by knowing the specific glands and salivary makeup that are altered at each time point.

3.5.2 Specific Protein Analysis of UWMS Pre and Post IMRT

PAS staining of UWMS glycoproteins demonstrated how mucin 5B and mucin 7 were both strongly affected by radiotherapy. Analysis of each mucin noted the increased concentration of mucin 5B and 7 at T1 in comparison to pre-IMRT (T0). This is all whilst maintaining the same levels of secretion for mucin 5B and 7, at all-time points. It should be noted that variance in data points for mucin 7 at T2, does not change the overall observation that the salivary structure would have fundamentally changed for the patients.

Past studies have proposed the possibility that the patients would have been able to feel the increased concentrations of mucin 5B and mucin 7, as increased levels of these mucins raise the viscosity of saliva and ultimately alter the rheological properties of saliva (Inoue et al., 2008).

In accordance with the data presented in this study, Randall et al., (2013) reported that during IMRT, mucin 5B concentrations in stimulated whole mouth saliva were increased in 20 HNC patients along with a significant reduction of salivary flow rate. However, that study only lasted 6 weeks, making it difficult to draw conclusions regarding protein variation post the

cancer treatment, and furthermore the Randall study, like most other studies that have assessed mucin concentrations, have utilised patients' stimulated whole mouth saliva likely due to difficulties in obtaining enough UWMS post radiotherapy in order to make valid comparisons.

Unlike Randall et al., (2013), some past studies have reported decreased secretion rate of mucin 5B in stimulated saliva collected from HNC patients (Almståhl et al., 2001; Dijkema et al., 2012). However, each study protocol differed, with Almståhl et al., (2001) comparing radiotherapy patients' stimulated saliva with those of healthy control samples, rather than the same patients prior to radiotherapy; Dijkema et al., (2012)utilised stimulated submandibular saliva of patients in two groups – comparing those with none/mild xerostomia and those with severe xerostomia. Although this in and of itself may be drawing inconsistence conclusions, as it has long been established that mucin 5B concentrations are associated with dry mouth reports by patients.

What we can infer from these results is that because the secretion rates were maintained and not changed for mucin 5B and 7, at all-time points, then the mucin secreting glands were spared, at least functionally, by IMRT in this patient cohort. Sublingual glands are the major contributors of both mucins, along with minor salivary glands, so it can be inferred that at least one of these glands were spared by IMRT in these patients.
It might be possible that the patients were capable of glandular recovery from the side effects of radiotherapy by T1, judging by their mucin secretion rates remaining similar to baselines, despite the fact that there was a reduced total protein secretion rate and salivary flow rate, this might be a mechanic compensation of the lack of total protein or due to an altered functionality as a result of a different glycoprotein structure, it would be important to assess salivary flow rate in minor salivary glands along with mucin 5b and 7 concentration and secretion rate. It is important to remark that there was no significant difference in concentration and secretion rate of these mucins regarding different tumour location in this group of patients which is why it is important to study different locations through the mouth including palatal, buccal and labial minor glands.

The increased concentrations of both mucins in UWMS post radiotherapy in this study may be connected to the xerostomia reported by patients, this was suggested by the significant association at time point 1 and 2 between dry mouth feeling reported with an increased

mucin 5B and 7concentration (Table 3.12 And 3.14) and mucins secretion rate in saliva observed at T1 and T2 (Table 3.3 and 3.5 respectively). Whilst the mucins alter the rheological properties of saliva (Inoue et al., 2008), there is also a further aspect of diminished lubrication of oral mucosa in the oral cavity as a result of the increased mucin 5B and mucin 7. There is a lack of information in relation to this phenomena in HNC patients, however it has been well established that the mucosal pellicle formation requires mucin- mucin interactions driven by mucin 1, which is secreted by minor salivary glands and it is expressed by epithelial cells membrane (Hannig et al., 2017). A lack of this protein will impair pellicle formation diminishing the possibility of binding mucin 5B and 7 to oral mucosa (Lynge Pedersen and Belstrøm, 2019), it would be therefore interesting to investigate the concentration of this protein in mucosal boundaries in future studies. Mucin 1 present in the surface of oral epithelium is defined as a membrane associated glycoprotein present mainly in the buccal and labial surfaces(Pedersen et al., 2018).

Another possible connection between dry mouth and an increased mucin concentration is an altered mucin molecular composition affecting protein carbohydrate proportion, resulting in an altered lubrication property and making mucins more vulnerable to proteolytic degradation (Takehara et al., 2013). The Chaudhury group found similar results in 2015 to this present study, in so far as patients were suffering from dry mouth, that mucin 5B and mucin 7 were at similar secretion levels, if not slightly higher. But they attributed signs of xerostomia, exhibited by patients despite the abundance of these moisture-retaining proteins, to a potential loss of the lubricating and retention/adhesion properties of saliva caused by structural changes in glycosylation ratio per unit of protein(Chaudhury et al., 2015).

Therefore, knowing that mucin 5B is amongst the main constituents of the mucosal pellicle, along with the IgA forming complex with mucin 7(Dawes et al., 2015; H. L. Gibbins et al., 2014; Vissink et al., 2010). IgA is the next logical bio-compositional aspect of saliva to study. Similarly, to the mucins, IgA secretion rate did not vary at any time points post radiotherapy in UWMS. This could mean that there were increased quantities of IgA in relation to total protein secretion rate, reflecting that some salivary glands were less affected by radiotherapy and maintained salivary production and compensated for the disfunction of other glands. A negative association between this protein and flow rate has been reported (Kugler et al., 1992; Närhi et al., 1994; Eliasson et al., 2005).

However, when comparing IgA concentrations, this theory might be disproven, as concentration levels of IgA also did not alter at any time point following IMRT. This has been in line with past studies that observed IgA concentrations in patients would either remain stable (Eliasson et al., 2005), or increased short term, returning to baseline in samples collected after one year (Funegård et al., 1994). More recently Richard et al 2017 studied 26 HNC patients Pre and Post IMRT and also reported no significant variation at 3-6 months, as well as at 12 months post IMRT in unstimulated parotid saliva when compared with baseline IgA concentrations. It is well known that minor salivary glands are a major source of IgA in the oral cavity (Sonesson et al., 2003; Eliasson et al., 2005; Eliasson and Carlén, 2010). As IgA secretions were unaffected by IMRT in this study, it can be inferred that the minor glands were spared, or at least functionally recovered by T1. This is similar to the previous mucins results that inferred that sublingual gland or again minor salivary glands were probably spared by IMRT treatments.

Having observed sublingual, as well as minor gland function, through mucins and IgA acting as possible functional biomarkers, α -amylase is the next protein to observe as it is the most abundant protein in saliva secreted by parotid glands (Carpenter, 2013b) as well as is the single most abundant protein in ductal saliva(H. L. Gibbins et al., 2014), serving as an indicator of serous gland function as well as reflecting any cell damage. A reduction in secretion rate of this protein would be an indicator of altered functioning of the parotid gland as a result of radiotherapy dose (Almståhl et al., 2001; Hannig et al., 2006).

The aforementioned reduction is precisely what occurred, with both α-amylase unit (measuring catalytic activity) and α-amylase secretion rate significantly reduced post IMRT, along with a significant association of this protein concentration and secretion rate with a reduced salivary flow rate at both time points (Table 3.7 and 3.8), showing that parotid glands were affected by IMRT, at T1 and T2, with parotid gland secretion capacity reduced as demonstrated by α-amylase. The reduced α-amylase concentration in saliva post radiotherapy is in accordance with findings of Almståhl et al., (2001), who found that the reduced concentration of α-amylase along with hyposalivation may indicate a loss of serous cell function. Similarly, De Barros Ponte et al 2004 found a significant reduction of salivary α-amylase activity, presented by units, post irradiation in stimulated whole mouth saliva, mainly secreted by parotid glands.

The recovery of amylase catalytic activity, as measured by units, in T2, which was to an extent that is statistically not-significantly different from baseline measurements, was not replicated in secretion rates as α-amylase secretion rate at T2 was still significantly lower than baseline rates. This could be associated with the slight salivary flow rate recovery at T2 reported in chapter 2 and corroborated by the significant association showed in this chapter at the same time point. This is keeping in line with similar findings in a non-cancer patient, whereby 77 subjects affected by rheumatological diseases were reported to have shown reduced α-amylase levels in unstimulated whole mouth saliva compared with controls (Helenius et al., 2005).

This recovery of α-amylase units to baseline levels is keeping in line with Hannig et al., (2006), who reported that there were no differences in α-amylase levels between 10 HNC patients treated with radiotherapy and controls. It is important to note that these patients underwent to IMRT between 6 and 12 months prior the sample collection, therefore the time frame of the sample collection is important because this current study observed an increase in α-amylase units per ml of saliva post 12 months of finished IMRT, as well as a slightly but not significantly increased secretion rate of this enzyme, therefore it can be inferred that Hanning et al's findings were perhaps closer to the 12 month point of the 6 to 12months time range.

Another known side effect of HNC radiotherapy, besides xerostomia, is stomatitis and in particular inflammation of the oral mucosa (Sciubba and Goldenberg, 2006), with more than two-thirds of radiation and chemotherapy-treated HNC patients developing severe mucositis, due to the cytotoxic effects of inflammation on epithelial tissue (Sonis, 2011; Epstein et al., 2012; Villa and Sonis, 2015). Albumin concentration in saliva can act as a good biomarker in detecting the presence of oral mucositis as it has been related to inflammatory process in salivary glands and serum leakage (Jensen et al., 2003).

The present study found that albumin concentration was statistically significantly higher in UWMS in patients post IMRT at T1, whilst secretion rate remained at a statistically non-significantly changed level. This indicates that the oral side effects of RT, particularly inflammation could have provoked epithelial disruption, making the oral mucosa very fragile causing plasma leakage and the direct side effect on salivary glands causing inflammatory reactions, as shown by the increase in albumin concentration.

However, at T2, albumin concentration was significantly reduced to levels similar to pre IMRT. Furthermore, secretion rate was also reduced at T2, being significantly lower than both time points 0 and 1, indicating a recovery of the oral mucosa over time. Moreover, the increased concentrations found at T1, could potentially be linked to the severe lack of saliva at that time point. However, this would be only 1 factor involved as salivary flow rates remained significantly low post 1 year (Chapter 2) whereas albumin concentrations returned to baseline levels.

Past studies, albeit ones who observed stimulated saliva rather than unstimulated, also observed increases in albumin concentrations in HNC patients post radiotherapy in comparison with controls (Almståhl et al., 2001; Eliasson et al., 2005). Whereas studies of non-cancer patients with systemic disease who are of similar age ranges have found salivary albumin concentration in stimulated saliva to also be significantly higher (Meurman et al., 2002). Clinically these hospitalized patients had worse oral health than outpatients along with a reduced salivary flow rate (Meurman et al., 2002; Zussman et al., 2007).

The hypothesis that as inflammation levels died down, albumin concentrations returned to baseline levels is further backed by studies that observed altered salivary profiles of heavy smokers with diseases, such as Nagler (2007), finding that IgG and albumin were reduced in smokers in comparison to non-smokers. This is explained by nicotine usage causing anti-inflammatory reactions in the oral cavity (McQuibban et al., 2000; Nagler, 2007).

Having observed sublingual, parotid, and minor glands functionality, through mucins, α - amylase and IgA acting as biomarkers respectively, the next protein to observe is cystatin S as it can act as a biomarker for submandibular glands, it is mainly secreted by submandibular glands (Baron et al., 2008; Martini et al., 2017).

This study found a severe reduction in concentrations levels of cystatin S at both time points 1 and 2, as well as a statistically significant reduction of secretion rates at T1 and T2, possibly indicating that IMRT affects the submandibular glands directly leading to reduced cystatin S production. Furthermore, the concentration and secretion rates were both significantly correlated with UWMS flow rate at both time points (Table 3.9 and 3.10 respectively). Submandibular glands are the biggest contributor of UWMS in terms of secretion and account for 60% of flow rates (Pedersen et al., 2018). Therefore, the severe reduction in flow rate,

combined with the severe reduction in concentrations levels and secretion rate of cystatin S, all combine to suggest that IMRT directly affected submandibular glands (Hawkins et al., 2018). This association between UWMS flow rate and cystatin S production has been previously explored (Martini et al., 2017), with studies that analysed Sjogren syndrome finding cystatin S was significantly decreased in patients, especially in those with hyposalivation (Peluso et al., 2007; Martini et al., 2017). However, another study of HNC patients reported no difference in cystatin S concentration post radiotherapy compared with controls (Hannig et al., 2006), whilst another study reported reduced salivary flow rate and decreased overall cystatin S in saliva from patients with dry mouth in comparison to controls, but increased levels of cystatin S on "*residual fluids on mucosal surfaces*" however these fluids "*showed little remaining reactivity for cystatin S*" (Pramanik et al., 2010)(pg. 249). Pramanik's findings on residual fluids suggest that the proteins were in an altered dormant state on the residual fluids and not directly produced by the submandibular gland, as it has been seen that minor salivary glands located in the oral mucosa and consisting of a small cluster of seromucous cells can produce cystatin S (Siqueira et al., 2008; Eliasson and Carlén, 2010). This could explain the dramatic reduction after 6 months of radiotherapy and the slight recovery at the one-year time point. It would be helpful to asses this protein concentration and secretion rate during and immediately post radiotherapy, as some studies have found variations in shorter term time points (Laheij et al., 2015), as well as assessing production from minor salivary gland saliva collection, whether they are from labial or buccal surfaces as minor salivary glands have been identified as producing small quantities of cystatin S(Siqueira et al., 2008; Eliasson and Carlén, 2010).

Analysis of PRPs could also be a good indicator for both parotid and submandibular glands, as PRPs, containing between 25-42% of proline amino-acids are secreted by parotid and submandibular glands (Carpenter, 2013b). The present study found no statistically significant change in PRPs levels at any time point in comparison to baseline. But this is not to diminish the previous inference that parotid and submandibular glands were affected by IMRT, as another study observing cancers also found no changes in PRPs whilst finding changes in other salivary composites such as α-amylase (Warner et al., 1985). Similarly, other studies utilising animals models have also found a lack of significant change in PRPs (Ann et al., 1987;

BANNISTER et al., 1989).This has been the first study of its kind to observe PRPs in humans post radiation therapy.

Finally, comparing statherin levels can give an overview of the overall oral cavity homeostasis, as statherin is a phosphoprotein protein secreted from parotid, submandibular and sublingual glands. Maintaining calcium homeostasis, statherin prevents its precipitation and crystal growth, as well as keeping saliva supersaturated in order to stimulate the remineralisation process (Carpenter, 2013; Hemadi et al., 2017).

Statherin concentration levels found not to be statistically significantly changed, similar to PRPs, at any time point in comparison to baseline. But once again, similar to PRPS, this is not to diminish the previous inference that submandibular glands were affected by IMRT, as similar past studies have also found no changes in statherin following biological Stress (Hannig et al., 2006; Laheij et al., 2015).

As Chapter 2 reported taste disturbances by patients, along with reduced salivary flow rate, it is also important to analyse the levels of CA VI, which has been shown to play a fundamental role in controlling taste sensation and taste bud growth, by facilitating the interaction between food particles and taste buds (Denny et al., 2008; Hunter, 2013).

Carbonic anhydrase levels were statistically significantly increased at T1, whilst the graph (Figure 3.18) demonstrating that secretion rates were reduced at T1, although not to a statistically significant extent. Whilst analysis of data at T2 showed that CA VI concentration levels had returned to those similar of controls, secretion was statistically significantly reduced at T2 in comparison to T0. Additionally, carbonic anhydrase concentration and secretion rate were significantly associated to taste alteration reported by patients at both time points (Table 3.16 and 3.17). These alterations in levels associated to taste acuity could go some way towards understanding the taste disturbances reported in chapter 2 which were significantly associated with a reduced flow volume , it has been showed that the lack of saliva reduces the moistening process leading to a reduced capacity of chemoreceptors to accept stimuli, causing a lack of gustatory response (Sciubba and Goldenberg, 2006).

Furthermore, these show an altered parotid and submandibular gland function, as CA VI is an enzyme secreted by parotid and submandibular glands. This backs up our previous findings of

α-amylase indicating that parotid glands were affected by IMRT and our findings of reduced cystatin S suggesting that IMRT directly affected submandibular glands.

A further element to consider, as a possible reasoning for why certain protein concentrations and secretion rates are providing similar outcomes, is that all patients received a similar radiation dose and fractioning for their cancer treatment and that their main tumour sites were close anatomically and the tumour stages were similar, among the patient group. Radiotherapy dose and fractioning regime seems to be the determinant factor regarding normal tissue response in this group of patients. Radiation susceptibility of salivary glands cells to damage, magnitude of response, functional recovery and regeneration of the gland are determined by a genetic component that needs to be further studied in the literature (Andreassen, 2010). A different explanation that has been reported in the literature is the exposure to radiation induced oxidative stress in cells which would cause cellular apoptosis and senescence. A prolonged exposition to oxidative stress due to radiation toxicity might harm adjacent non-irradiated cells by communication through secreted factors this is known as a Bystander effect (Kadhim et al., 2013; Mothersill et al., 2019).

However, it is not possible to draw a definitive conclusion from reviewing literature regarding salivary composition changes post radiotherapy due to limited number of patients recruited for past studies, wide range of molecules investigated and short follow-up period in past studies, with the only consensus being that of salivary flow rate reduction post radiotherapy. Past studies have found IMRT to not fully spare tissues surrounding target area, when measured in terms of salivary flow rate (Dijkema et al., 2008), xerostomia (Dijkema et al., 2010), and other oral symptoms such as hyposalivation in general (Nutting et al., 2011; Nguyen et al., 2018). However, the clinical relevance of such initial findings changes depending on the function of each protein that is altered (Chao et al., 2001; Jawad et al., 2015). Therefore, the present study analysed several salivary bio-composites individually in order to evaluate the effects of IMRT on each individual gland and the association of this proteins with clinical altered symptoms. That analysis of biomarkers for individual glands , made the unique finding that although IMRT can spare some tissues, it is not entirely proof, with parotid and submandibular glandular function affected in particular, as evidenced by CAVI, α-amylase and cystatin S acting as biomarkers, whilst functional markers for sublingual and minor salivary glands showed them to be spared from IMRT or functionally recovered,

being capable of secrete certain proteins. This reinforces the findings of past studies that found IMRT does not fully spare tissues when measured as xerostomia or symptoms (Dijkema et al., 2010; Nutting et al., 2011), rather than glandular functions. Whilst others have shown how altering certain protein compositions will increase the possibility of breaking the equilibrium between microbial species in the oral cavity, reducing remineralisation, increasing erosion, and reducing food clearance process resulting in a high caries risk (Carpenter, 2013b; H. L. Gibbins et al., 2014; Kilian et al., 2016; Hannig et al., 2017). The direct causations that IMRT may have on such oral symptoms through its effects on salivary glands, was measured using a longitudinal panel data statistical model to assess their associations, that have not previously been performed for HNC patients. Therefore, it was imperative to analyse these links between the aforementioned factors, because it is well known that oral radiotherapy side effects reported previously might be connected to an altered salivary quality observed in this study, as a result of salivary gland hypofunction post IMRT (Dijkema et al., 2012; Richards et al., 2017). Indeed, this chapter observations were able to reflect salivary dysfunction, moreover, altered proteins concentration and secretion rate could be a mirror of each salivary gland specially taking in consideration the major UWMS contributors submandibular and sub lingual. It could be speculated that recovered minor gland might be trying to compensate reduced function. There is no literature regarding these gland function in head and neck cancer patients related to radiation therapy threshold, flow rate and composition. However there are studies that showed an altered secretion rate of minor saliva and mucosal pellicle protein composition in patients presenting xerostomia (Pramanik et al., 2010; Chaudhury et al., 2015; Hannig et al., 2017).

Overall, this study concludes that salivary flow and composition showed a significant, transient reduction in quantity and quality, following IMRT. However, some of the patients began to recover to normal levels at T2, 12 months post IMRT, there were reported significant associations among protein composition, secretion rate and this reduced volume of vital fluid as well as dry mouth feeling reported by patients and taste acuity.

Another important finding was that the primary tumour location did not affect the protein secretion rate and concentration in saliva in this group of patients in line with the past studies. The potential clinical relevance of these findings needs to be further investigated regarding oral care protocols improvements, oral risk assessment (pre and post IMRT) and personalized

monitoring of these conditions progression in order to improve this patient's quality of life. Finally, the possibility of developing novel therapies to help to reduce salivary gland dysfunction, induce recovery of function, along with promoting the use of IMRT capable to spare the submandibular and mucosal minor salivary glands without underdosing target tissues.

Chapter 4 Oral mucositis

4.1 Introduction

Chapters 2 & 3 previously reported the clinical outcomes as well as the biochemical salivary protein compositional variations following radiation treatments for head and neck cancers, specifically when localised near salivary glands and oral mucosal epithelium.

With patients undergoing radiotherapy for cancer treatment presenting multiple functional impairments and clinical symptoms, usually as a side-effect of the treatments, it becomes more important to understand the relationships between their health and any markers that may indicate early onset of upcoming side-effects (Fan, 2007; Raber-Durlacher et al., 2010; Münstedt et al., 2019), even more if these biomarkers can be identified prior to radiotherapy treatment. With saliva being the first line of defence in the oral cavity to protect tissues, saliva composition is essential not only to protect the oral cavity through lubricating and coating the epithelial surface (Asikainen et al., 2017), but also to act as a potential indicator for overall health of the salivary glands and the oral cavity's homeostasis.

One of the most common side effects, along with xerostomia, is mucositis, a morbid condition that occurs as frequently as 60-85% of cancer treatment patients (Sonis, 1998, 2004a; Villa and Sonis, 2015). Despite the high incidence rate of oral mucositis during cancer therapy and its clinical impact on patients and economic effect for healthcare providers, there is a lack of adequate treatment to reverse, control or prevent this side effect. Furthermore, the condition represents not only a problem for patients but also for clinicians(Rieger et al., 2012; Gao et al., 2015) with its management placing a strain on healthcare systems by increasing the financial burden of healthcare costs in comparison to the patients without oral ulceration (Sonis et al., 2001; Vera-Llonch et al., 2006), increasing the expenses as a result of additional medical attention, extended hospitalisation, increasing risks of secondary infections and lowering quality of life due to implementing special diets and parenteral feeding (Sonis, 2011; Villa and Sonis, 2016; McCullough, 2017). Therefore, from both economic and patient healthcare delivery perspectives, it could be a critical turning point for management of mucositis if early detection and/or prediction was feasible and implemented (Fan, 2007; Raber-Durlacher et al., 2010).

This study used the clinical skills of physicians from the Guy's and St Thomas' NHS oncology group to visually assess mucositis levels using the WHO mucositis scale, at each time point of the experiment (prior to; 6 months and 1 year post-radiotherapy). The findings were analysed in comparison to salivary proteins and mucins, in order to not only assess any relationships between the two elements of saliva and mucositis, but in order to also form a capacity to predict the severity of oral mucositis, by allowing salivary composition to act as a biomarker prior to radiotherapy, as demonstrated in Figure 4.1.

The WHO toxicity scale documents the severity and prevalence of mucositis, with the scale being one of the most commonly used in order to monitor this condition. However, there is no universal scale for measuring mucositis, with this being one of the biggest drawbacks in oral mucositis research as at the moment of writing this thesis. Other scales used include the Radiation Therapy Oncology Group scale (RTOG) and European Organisation for Research and Treatment of Cancer scale (EORTC). Whilst RTOG / EORTC are focused on patients' levels of pain and suffering, in order to measure the necessity of analgesia, the WHO toxicity scale focuses on patients' functionality in relation to the capacity to eat solid food (Sciubba and Goldenberg, 2006; Sroussi et al., 2017). However, these are all subjective measurements, with evaluative outcomes dependent on a physician's clinical experience and training in order to clearly define categories on each relevant scale.

The lack of a clear universal scale might act as a further barrier to development of a definitive predictive system for gradings of mucositis, however this study first focuses on establishing whether or not a clear predictive molecule for mucositis can be identified prior to radiotherapy treatment. If a biomarker is found, with its specificity and sensitivity rate of predictive accuracy analysed, then a further step can be taken to analyse each grading of mucositis against the diagnostic ability of predictions using a Receiver Operating Characteristic curve, or ROC curve. ROC curves are used in order to clearly plot the diagnostic ability of a binary classifier in cases where discrimination thresholds are varied (in this case being the ability to accurately predict oral mucositis in cases where salivary protein thresholds vary).

If such a finding can be identified, analysed and proven to associate against varying severities of mucositis, then this not only presents an opportunity to create a clear identifier to prevent cancer treatment interruption (Turner et al., 2013) but also the opportunity to create

personalised preventative care regimes in order to reduce the debilitating effects on mucositis, prior to the development of severe oral mucositis symptoms (Normando et al., 2017).

Analysis overview

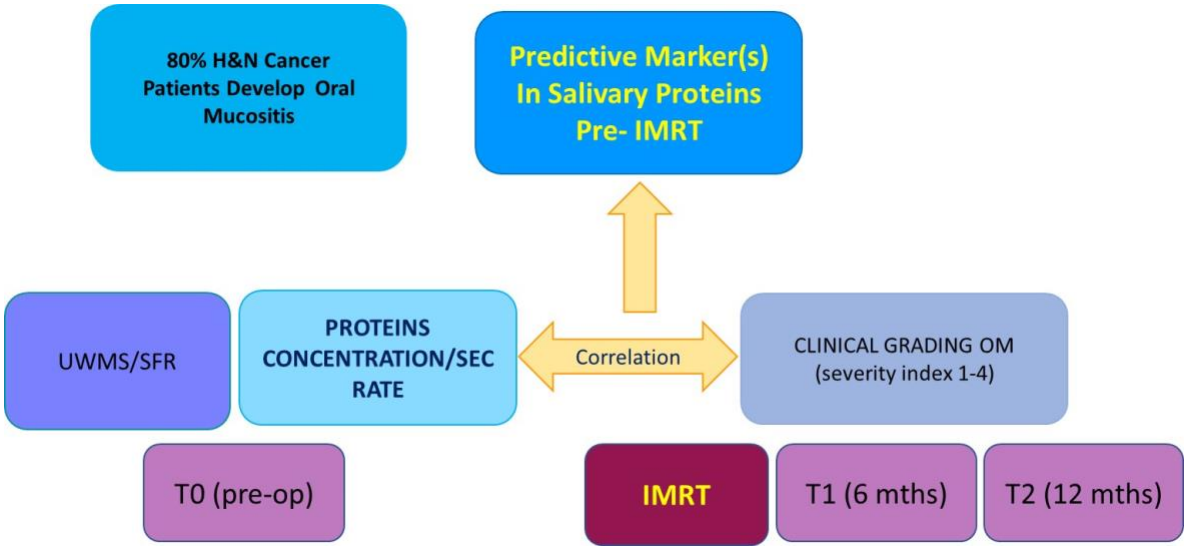


Figure 4. 1 Identifies the clinical problem and the process followed to find a possible marker of oral mucositis severity prediction.

4.2 Aim

The aim of this study focuses on establishing a predictive model for mucositis prior to radiotherapy treatment by analysing the possible associations between salivary protein concentration and secretion rate and clinical outcomes.

Null Hypothesis: There is no longitudinal association between IMRT-induced changes in salivary markers with oral mucositis severity.

There are no pre-operative salivary indicators for potential risk prediction of the severity of mucositis suffered during therapy

Objectives

To associate salivary protein concentration and secretion rate pre and post- IMRT with the clinical assessment of mucositis.

To assess specific proteins in saliva pre-IMRT as a potential risk predictor of the presence of mucositis suffered during therapy in head and neck cancer patients.

To assess specific proteins in saliva pre-IMRT as a potential risk predictor of severity of mucositis suffered after therapy in head and neck cancer patients.

4.3 Material and Methods

4.3.1 Patient Recruitment

Forty HNC patients were recruited in line with the Helsinki declaration with full, written informed consent gained. The study was approved by the North of Scotland Research Ethics Service (NRES) Committee foundation in October 2016 (16/NS/0116), the Health Research Authority NHS (IRAS Project ID:199100) and the patient recruitment letter is appended (Appendix no.1).

Recruitment was carried out prior to IMRT (T0) at Guy's Dental Hospital, London in the Special Care Dentistry Unit. Patients were followed up six months post-IMRT (T1) and 12 months post-IMRT (T2). Forty patients were recruited at T0, 38 patients were seen at T1 and 34 patients at T2 respectively.

4.3.2 Saliva Sample Collection

All saliva samples were collected from the patients by drooling method at every time point, and flow rate was assessed as described in detail in Chapter 2. One hundred and eleven saliva samples were collected and analysed.

4.3.3 Sample Analysis

Samples were analysed for total protein content using the Bicinchoninic Acid Assay (BCA), targeted protein concentration by Enzyme-Linked Immunosorbent Assay (ELISA), Periodic Acid Schiff (PAS) staining, Coomassie Brilliant blue and α-amylase activity by the standard kinetic enzyme assay (Salimetrics). The assays were carried out as set out in the manufacturer's guidelines.

Prior to biochemical analysis UWMS samples were centrifuged at 10000 RPM for 3 mins. The supernatant was separated to be used to analyse protein content and the pellet was preserved to perform DNA extraction.

4.3.3.1 Protein Analysis Concentration and Secretion Rate

In order to determine the salivary protein composition, the total protein content /secretion rate, specific protein concentrations and their secretion rates were analysed. Nine proteins (mucin 5B, mucin 7, IgA, α-amylase , albumin, cystatin S, carbonic anhydrase VI, acidic proline rich protein and statherin) were selected due to their relationship to host defence, antimicrobial, bacterial adherence, colonization, mucosal pellicle, enamel pellicle, viscoelastic, rheological properties, lubrication, and remineralization.

In summary, mucin 5B, mucin 7, IgA, α-amylase , albumin, cystatin S, carbonic anhydrase VI, acidic proline rich protein and statherin are all salivary proteins related to caries development, xerostomia, taste alteration and oral mucositis before and after radiation therapy in the reported longitudinal clinical study (12 months).

4.3.3.2 Oral Mucositis Assessment

In the study clinical assessment of oral mucositis was performed by the oncology team during and after the radiotherapy using the World Health Organization (WHO) oral toxicity scale (World Health Organization, Handbook, 1979).

This grading system is based on a clinical examination of the oral cavity, in the case of oral mucositis there were added three extra time points due to the acute nature of this toxicity that appears once the cancer treatment started.

Clinical assessment was recorded during radiotherapy and after 2 weeks, 6 weeks 3 months and the regular time points 6 months (T1) and 12 months (T2).

4.3.4 Statistical Analysis

Random effects Generalized Least Squares linear regression.

A random effects linear regression analysis in a longitudinal panel was used to analyse the data obtained from the patients over time. Panel refers to a group of subjects that were studied recurrently over time in order to determinate the association between salivary

proteins and oral mucositis development at T1 (6 months post-IMRT) and T2 (12 months post-IMRT) in comparison to T0 (pre-IMRT).

Receiver Operating Characteristic (ROC) curve

ROC analysis was used to analyse sensitivity and specificity of certain salivary proteins to predict the development and severity of oral mucositis during IMRT prior to commencement of the radiotherapy treatment.

The true positive rate (sensitivity) is plotted in function of the false positive rate (100-specificity) for different cut-off points of a parameter. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold.

ROC curve is an analysis tool used to evaluate the performance of a diagnostic test, based on sensitivity and specificity for every possible cut point for a test. It is a plot of the true positive rate (sensitivity) in function of the false positive rate (100-specificity) for different cut-off points of a parameter. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. In addition, it would reveal the area under curve (AUC) which is a measure that represent the whole performance of the test being the sensitivity average value for all specificity values, demonstrating how well a parameter can discriminate between disease and non-disease subjects. The higher the AUC, the better the model is at distinguishing between patients with or without disease. Therefore, a test with a higher accuracy will have a higher AUC, the lower limit should be 50% in order to keep an optimal test performance to distinguish between patients that present the disease from those without the disease and not relying on pure chance. AUC is not dependent on disease prevalence (Metz, 1978; Bradley, 1997; Obuchowski, 2003; Park et al., 2004; Zhou et al., 2011). Sensitivity is defined as a true positive rate (TPR) expressed as a percentage, being the proportion of positive samples that present with the disease and specificity true negative rate (TNR) expressed in percentages being the proportion of negative samples that were correctly classified as non-disease subjects (Tharwat, 2018).

A test sensitivity and specificity is chosen in the context of the type of disease to be diagnosed; for potentially life threatening diseases the cut point range for a positive test would provide a high sensitivity in order to reduce false negative subjects, as a false negative test could have serious inplications for survival of the patients (Park et al., 2004).

All analyses were carried out using STATA 15.1 (College Station, Texas USA, www.stata.com), GraphPad Prism 8 software (La Jolla California USA, www.graphpad .com) and Microsoft Excel 2018. Results were expressed as a percentage for sensitivity, specificity and AUC, cut point value for concentration or secretion rate of each protein as a threshold. P value was set at <0.05.

4.4 Results

4.4.1 Longitudinal Associations Between Oral Mucositis Outcomes and Salivary Proteins Concentration and Secretion Rate Post IMRT at 6 Months (T1) and 12 Months (T2) by using Random effects Generalized Least Squares linear regression.

This study found that between the 31 patients who developed mucositis during IMRT, there were significant associations had formed, beginning to associate total protein concentration and oral mucositis at T1(*p=0.009)* (Table 4.1). Furthermore, there appeared to be statistically significant associations formed between oral mucositis and total protein secretion rate at Time Point 1 (*p=0.017*) (Table 4.2).

When comparing associations between oral mucositis assessments and mucins 5B and 7, although there were no statistically significant associations formed in comparing concentrations of the mucins with oral mucositis at both time points post IMRT, there were statistically significant associations for mucin 5B's secretion rate at Time Point 1 (*p=0.017*) as well as mucin 7's secretion rate at Time Point 1 (*p=0.015*) (Table 4.4 and 4.6)

In keeping with the pattern of significance at Time Point 1, α-amylase units and secretion rate were both associated with oral mucositis assessments at statistically significant levels at Time Point 1 (*p=0.040 and p=0.037* respectively). Similarly, albumin concentration and secretion rate were both significantly associated to oral mucositis presence at Time Point 1 (*p= 0.007 and p= 0.014* respectively), whereas for all comparisons of oral mucositis with Albumin and α-amylase, there were no statistically significant associations at Time Point 2.

Cystatin S concentration was significantly associated with oral mucositis at Time Point 1 (*p=0.039),* keeping in line with cystatin S secretion rate which appeared to have a borderline associated at Time Point 1 *(p=0.057*). The associations of cystatin, α-amylase , albumin, mucins 5B and 7 and oral mucositis all becoming statistically significant at Time Point 1 is in keeping with the results of Chapter 2 that found that following IMRT, between Time Points 0 and 2, there were significant drops in the number of patients that presented with oral mucositis (*p=<0.0001*).

Immunoglobulin A concentration uniquely presented a significant association at both Time Points 1 & 2 in relation to oral mucositis (*p=0.007 and p= 0.03* respectively). IgA secretion rate

was also significantly associated at Time Point 1 (*p= 0.009*) with a borderline association to oral mucositis at Time Point 2 (*p=0.052*).

Table 4.1 Oral mucositis assessment in the oral cavity and total protein concentration in unstimulated whole mouth saliva (UWMS) at T1 (6 months post-IMRT) and T2 (12 months post-IMRT) in comparison to T0 (pre-IMRT).

Oral Mucositis /TPC	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T 0	0.027	0.052	-0.0002	0.055
T 1	**0.153**	**0.009**	0.038	0.267
T 2	0.071	0.237	-0.046	0.188

Table shows the significant relation between oral mucositis and total protein concentration (TPC) in UWMS after IMRT at Time Point 1.

Table 4.2 Oral mucositis assessment in the oral cavity and total protein secretion rate in unstimulated whole mouth saliva (UWMS) at T1 (6 months post-IMRT) and T2 (12 months post-IMRT) in comparison to T0 (pre-IMRT).

Oral Mucositis/ TP SR	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T 0	0.0182	0.667	-0.064	0.101
T 1	0.156	**0.017**	0.0281	0.283
T 2	0.097	0.111	-0.022	0.217

Table shows the positive and significant relation between oral mucositis and total protein secretion rate in UWMS after IMRT at Time Point 1.

Table 4.3 Oral mucositis assessment in the oral cavity and mucin 5B concentration in UWMS at T1 (6 months post-IMRT) and T2 (12 months post-IMRT) in comparison to T0 (pre-IMRT).

Oral Mucositis/ Mucin 5B	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	0.0005	0.157	-0.0002	0.0013
T1	0.078	0.272	-0.0617	0.219
T2	0.036	0.605	-0.1011	0.174

Table shows non-significant relation between oral mucositis and mucin 5B concentration in UWMS after IMRT at Time Point 1 and Time Point 2.

Table 4. 4 Oral mucositis assessment in the oral cavity and mucin 5B secretion rate in UWMS at T1 (6 months post-IMRT) and T2 (12 months post-IMRT) in comparison to T0 (pre-IMRT).

Oral Mucositis/ Mucin 5B SR	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	-0.000046	0.954	0-.0016	0.0015
T1	0.138	**0.017**	0.0247	0.252
T2	0.088	0.139	-0.0289	0.206

Table shows the significant relation between oral mucositis and mucin 5B secretion rate in UWMS after IMRT at Time Point 1.

Table 4.5 Oral mucositis assessment in the oral cavity and mucin 7 concentration in UWMS at T1 (6 months post-IMRT) and T2 (12 months post-IMRT) in comparison to T0 (pre-IMRT).

Oral Mucositis/ MUC 7	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	0.0038	0.0001	0.0025	0.0051
T1	0.051	0.330	-0.0512	0.153
T2	0.072	0.155	-0.0275368	0.172

Table shows no significant association after IMRT at Time Point 1 and 2 between oral mucositis and mucin 7 concentration in UWMS after IMRT.

Table 4.6 Oral mucositis assessment in the oral cavity and mucin 7 secretion rate in UWMS at T1 (6 months post-IMRT) and T2 (12 months post-IMRT) in comparison to T0 (pre-IMRT).

Oral Mucositis/MUC 7 SR	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	0.0007	0.381	-0.0009	0.0025
T1	0.135	**0.015**	0.026	0.244
T2	0.079	0.167	-0.033	0.193

Table shows significant relation between oral mucositis and mucin 7 secretion rate in UWMS after IMRT at Time Point 1.

Table 4.7 Oral mucositis assessment in the oral cavity and α-amylase unit in UWMS at T1 (6-months post IMRT) and T2 (12 months post-IMRT) in comparison to T0 (pre-IMRT).

Oral Mucositis/ α-amylase U	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	0.0003	0.266	-.0002	.0008
T1	0.107	**0.04**	.004	.208
T2	0.069	0.177	-.031	.169

Table shows a significant relation between presence of mucositis and α-amylase unit in UWMS after IMRT at Time Point 1.

Table 4.8 Oral mucositis assessment in the oral cavity and α-amylase unit secretion rate in UWMS at T1 (6 months post-IMRT) and T2 (12 months post-IMRT) in comparison to T0 (pre IMRT).

Oral Mucositis/α-amylase U SR	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	0.0008	0.290	-.0006	.002
T1	0.118	**0.037**	.007	.229
T2	0.088	0.104	-.018	.195

Table shows a significant relation between oral mucositis and α-amylase unit secretion rate in UWMS after IMRT at Time Point 1.

Table 4.9 Oral mucositis assessment in the oral cavity and albumin concentration in UWMS at T1 (6 months post-IMRT) and T2 (12 months post-IMRT) in comparison to T0 (pre-IMRT).

Oral Mucositis/Albumin	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	-0.0002	0.500	-0.0009	0.00045
T1	0.151	**0.007**	0.040	0.262
T2	0.064	0.264	-0.048	0.177

Table shows a significant relation between presence of mucositis and albumin concentration in UWMS after IMRT at Time Point 1.

Table 4.10 Presence of mucositis in the oral cavity and albumin secretion rate in UWMS at T1 (6 months post-IMRT) and T2 (12 months post-IMRT) in comparison to T0 (pre-IMRT).

Oral Mucositis/Albumin SR	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	-0.0007	0.466	-0.0028	0.0012
T1	0.136	**0.01**	0.027	0.245
T2	0.056	0.344	-0.060	0.172

Table shows a significant relation between presence of mucositis and albumin secretion rate in UWMS after IMRT at Time Point 1.

Table 4.11 Presence of mucositis in the oral cavity and cystatin S concentration at T1 (6 months post-IMRT) and T2 (12 months post-IMRT) in comparison to T0 (pre-IMRT).

Oral Mucositis/Cystatin S	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	0.002	0.638	-0.008	.012
T1	0.171	**0.039**	0.008	.334
T2	0.125	0.103	-0.025	.277

Table shows the positive and significant association between presence of mucositis and cystatin S concentration in UWMS after IMRT at Time Point 1.

Table 4.12 Presence of mucositis in the oral cavity and cystatin S secretion rate at T1 (6 months post-IMRT) and T2 (12 months post-IMRT) in comparison to T0 (pre-IMRT).

Oral Mucositis/ Cystatin S SR	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	-0.0003	0.966	-0.0147	.014
T1	0.143	**0.057**	-0.004	.291
T2	0.105	0.161	-0.041	.253

Table shows the positive and significant association between presence of mucositis and cystatin S secretion rate in UWMS after IMRT at Time Point 1.

Table 4. 13 Presence of mucositis in the oral cavity and IgA concentration in UWMS at T1 (6 months post-IMRT) and T2 (12 months post-IMRT) in comparison to T0 (pre-IMRT).

Oral Mucositis/IgA	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	-0.00067	0.597	-0.003	0.001
T1	0.156	**0.007**	0.041	0.271
T2	0.140	**0.030**	0.013	0268

Table shows a significant association between presence of mucositis and IgA concentration in UWMS after IMRT at Time Point 1 and at Time Point 2.

Table 4.14 Presence of mucositis in the oral cavity and IgA secretion rate in UWMS at T1 (6 months post-IMRT) and T2 (12 months post-IMRT) in comparison to T0 (pre-IMRT).

Oral Mucositis/IgA SR	**Coefficient**	**P**	**[95% Conf.**	**Interval]**
T0	0.004	0.153	-0.001	0.0101
T1	0.154	**0.009**	0.0385	0.269
T2	0.126	**0.052**	-0.0009	0.254

Table shows the positive and significant association between presence of mucositis and IgA secretion rate in UWMS after IMRT at Time Point 1 and a borderline significant relation at Time Point 2.

4.4.2 Receiver-operating characteristics curves (ROC)

Following analysis of associations between assessments of oral mucositis with different protein concentrations and secretion rates at all time points, it is possible to take the next step of analysing the presence of mucositis (as well as grading of oral mucositis) against the previous associations, with the specificity and sensitivity rate of predictive accuracy analysed, allowing a diagnostic ability of predictions using a ROC curve.

The summary of analysis of sensitivity (true positives) and specificity (true negatives) measured before IMRT, as a point of prediction of mucositis onset, revealed that α-amylase unit concentration can be an accurate predictor of oral mucositis onset during IMRT on occasions where α-amylase units are more than 30.14 pre-IMRT (when measured in units per ml of unstimulated whole mouth saliva).

The analysis of sensitivity and specificity measured before IMRT, as a point of prediction of mucositis severity, revealed that mucin 5B concentration and secretion rates assessed before cancer treatment can be very accurate predictors of oral mucositis severity grades 1 & 2, showing correct classification rates of 92.31%, with an area under the ROC curve of 90.91% for concentration and 86.36% for secretion rate.

Furthermore, analysing the ROC curve of results 2 weeks after IMRT revealed that α-amylase unit concentration can also be an accurate predictor of oral mucositis grades 1 & 2, showing correct classification rates of 87.5%, with an area under the ROC curve of 91.67%.

4.4.2.1 Analysis of Salivary Proteins Collected at Time 0 as A Molecular Predictor of Mucositis Development During Radiotherapy

Table 4.14 Oral mucositis presence during radiotherapy.

ROC curve analysis of proteins in UWMS	Protein at Time 0	Cut point	Sensitivity	Specificity	Correctly Classified	AUC
Mucositis present during IMRT	α-amylase unit /ml of UWMS	≥ 30.14	**92.59%**	**100.00%**	**93.10%**	**92.50%**

4.4.2.2 Analysis of Salivary Proteins Collected at Time 0 as A Molecular Predictor of Oral Mucositis Severity During Radiotherapy

Table 4.15 Oral mucositis severity during radiotherapy.

ROC curve analysis of proteins in UWMS	Protein at Time point 0	Cut point	Sensitivity	Specificity	Correctly Classified	AREA
Mucositis severity (1&2) during IMRT	MUC 5B ug/ml	≥16.16	**100.00%**	50.00%	92.31%	90.91%
	MUC 5B ug/min	≥4.65619	**100.00%**	50.00%	92.31%	86.36%
Mucositis grade 2 during IMRT	**MUC 5B ug/ml**	≥ 68.64	64.29%	73.68%	69.70%	62.22%
Mucositis severity 3 during IMRT	Total protein concentration mg/ml	≥ 1.94487	**90.00%**	50.00%	62.50%	62.27%
	TPC sec rate mg/min	≥1.134	70.00%	59.09%	62.50%	65.45%

4.4.2.3 Analysis of Salivary Proteins Collected at Time 0 as A Molecular Predictor of ***Oral Mucositis Severity Post IMRT Radiotherapy***

Table 4.16 oral mucositis severities of 2 weeks and 6 weeks post-IMRT

ROC curve analysis of proteins in UWMS	Protein at Time point 0	Cut point	Sensitivity	Specificity	Correctly Classified	AUC
OM grade **1 & 2** **2weeks** post IMRT	α-amylase U/ ml	≥22.77	**100.00%**	**50.00%**	**87.50%**	**91.67%**
OM grade **2** **2 weeks** post-IMRT	MUC 5B secretion rate ug/min	≥ 27.97	**76.92%**	63.16%	68.75%	65.18%
	MUC 7 secretion rate ug/min	≥ 1.103	57.14%	54.55%	55.56%	57.47%
	α-amylase U/ml	≥56.84	**92.86%**	40.00%	61.76%	65.36%
	α-amylase U/ml	≥ 78.14	71.43%	55.00%	61.76%	65.36%
	α-amylase secretion rate U/min	≥31.25	**92.86%**	60.00%	73.53%	**70.71%**
	IgA secretion rate ug/ml	≥4.090	**75.00%**	60.00%	65.63%	57.5%
	Albumin secretion rate ug/min	≥ 16.33	64.29%	54.55%	58.33%	56.17%
OM grade **2** **6 weeks** post-IMRT	α-amylase U/ml	≥108.619	**85.71%**	47.06%	58.33%	58.82%
	α-amylase secretion rate U/min	≥42.7959	71.43%	41.18%	50.00%	60.50%
	IgA secretion rate ug/min	≥4.177	66.67%	58.82%	60.87%	54.9%

4.5 Discussion

Over the past two decades, the diagnostic value of oral fluids has become recognised, in particular for oral diseases, such as periodontal diseases (Miller et al., 2006), assessing caries risk (Spielmann and Wong, 2011), and cancers (Gualtero and Suarez Castillo, 2016). More recently, the combination of emerging biotechnologies has allowed salivary diagnostics to extend beyond the traditional range to predict wholly physiological risks to the entire oral cavity.

As set out in Chapter 1, the carefully regulated homoeostasis of a multitude of proteins in the oral environment provide functional lubrication and dehydration, as well as protection from colonisation by pathogenic organisms. Previous studies have established that the volume of saliva and its composition are vital to maintain oral homeostasis and reduce the possibility of developing oral diseases, such as mucositis (Carpenter, 2013b; H. L. Gibbins et al., 2014; Kilian et al., 2016; Hannig et al., 2017).

This study has been the first, as of this writing, to identify potential links between salivary biomarkers and the potential onset of oral mucositis, within the largest longitudinal and most comprehensive biochemical analysis of saliva from head and neck cancer patients spread across 1 year.

This is of particular note, with oral mucositis being a very frequent side effect of head and neck cancer radiation therapy treatment, occurring as frequently as 60 – 85% of patients (Fan, 2007; Raber-Durlacher et al., 2010; Münstedt et al., 2019), causing a huge measurable impact on patient welfare and the overall healthcare system (Vera-Llonch et al., 2007; Rieger et al., 2012). Therefore, in cases such as this where a patients quality of life can be so severely impacted (Villa and Sonis, 2016; McCullough, 2017), not only is early detection vital, but analysis of salivary fluids presents a great opportunity to create personalised preventive care regimes in order to mitigate potential suffering using non-invasive sample collection and simple processing.

Whilst Chapter 3 found that CA VI, mucins, α-amylase and cystatin S an act as biomarkers for glandular function, revealing whether or not particular salivary glands are spared from radiotherapy toxicity during IMRT, here this process can be taken one step further to analyse

the same salivary compositional collection for use as a preventative diagnostics tool – essentially identifying the accuracy of predicting future onset of oral mucositis, as well its potential severity.

The data presented in this study showed the radiotherapy effects on salivary glands resulting in a decreased oral function that remains after one year of completed IMRT. This study contributes to oral mucositis research by identifying the difference in protein signature of UWMS from HNC patients post-IMRT up to one year after the treatment has finished along with the onset of side effects, suggesting the possibility of an association between clinical outcomes regarding oral mucositis onset /severity and saliva functional and protective biochemical composition including total protein concentration and secretion rate followed by specific salivary proteins mucin 5B, mucin 7 and IgA as a part of the mucosal protection barrier and hydration layer, along with α-α-amylase that play a role in bacterial colonization, cystatin S as an antibacterial protein and part of pellicle, and albumin as marker of inflammatory processes. All these proteins were taken into account as patient-related contributors of developing this adverse side-effect and its severity.

Therefore, a comprehensive analysis of these molecular changes in saliva and the association with oral mucositis might allow the targeting of certain proteins that might be suitable as a predictor for early identification of patients prone to develop severe mucositis. In addition, this would allow a prompt and frequent monitoring of this side effect and in the long run would help to provide information to predict the risk of every patient of developing oral mucositis and customize their care to avoid reaching the highest severities and finally to reduce the chance of cancer treatment interruptions.

This chapter revealed significant positive associations when measuring total protein secretion rates, as well as concentrations, with oral mucositis at T1. This is of particular note as the findings in chapter 3 revealed that total protein secretion rate parameter was significantly reduced post-radiotherapy along with a reduced total concentration at T1, thus altering salivary properties and ultimately salivary flow rates. Salivary flow rate (negatively) and total protein secretion rate (positively) are associated with xerostomia reported by patients as previously reported in Chapter 2 and 3 and xerostomia was also found to be potentially positive and significantly associated with oral mucositis in Chapter 2. In summary, this demonstrates the positive and strong association between salivary gland hypofunction

caused by IMRT and oral side effects and the influence that this altered fluid might have on onset and severity regarding oral mucositis as well as in an altered mouth feel due to a reduced volume along with an altered salivary quality.

This all links to the previous findings that whilst total protein secretion rate was significantly reduced post-radiotherapy, a reduced concentration was associated to diminished salivary flow rate along with oral mucositis onset and development were increasing comparatively, with approximately 20% of all patients presenting mucositis at Time Point 1.

Furthermore, when analysing the protein composition on an individual basis, significant associations were found between oral mucositis and mucin 5B secretion rates, as well as mucin 7 secretion rates, all at Time Point 1. As previously shown in Chapter 3, the analysis of changes to mucin 5B and 7 revealed increased concentrations, which have been shown to affect saliva's viscoelasticity, effectively making saliva have a "*sticky effect*" and reducing its functional value (Veerman et al., 1989; Ligtenberg and Almståhl, 2015). The combination of these findings point to the previous links associated with altered flow rates and salivary composition that can negatively influence the occurrence and severity of mucositis (Eliasson et al., 2009), especially in head and neck cancer patients undergoing radiotherapy(Sonis, 2004a, 2011, 2013; Villa and Sonis, 2016; De Sanctis et al., 2016; Normando et al., 2017; Franco et al., 2017; Orlandi et al., 2018). In order to maintain mucosal integrity oral surfaces should be covered by a strongly retained, protective protein layer which can be able of providing adequate hydration and lubrication which are the key function of both mucins. Thus, the increased mucin 5B and 7 concentrations had increased viscosity, reducing mucosal wetness capability and leaving oral surfaces unprotected. In order to corroborate this assumption, it should be investigated the presence of these molecules adjacent to oral mucosa, by collecting residual saliva.

Another possibility that could be explored in future studies is the underlying causalities behind the alterations of mucins, with a possibility that the radiotherapy has caused an alteration of the cellular structure of the superficial epithelial cells that are decorated with numerous membrane ridges, termed microplicae (MPLs). The MPL structure is typical of the epithelial surfaces that are covered with protective mucus (Asikainen et al., 2015) and alterations in MPLs affect the adhesion of mucins to oral mucosa and ultimately suggest a loss of mucin retention (Dijkema et al., 2012; Asikainen et al., 2017). A recent study, albeit one

performed on animals, not humans, has observed that radiotherapy does cause structural modification, even one year after radiotherapy, to MPL (Asikainen et al., 2017). The MPL is a fundamental part of the interaction of mucins to form and hold a highly hydrated gel mucus layer to cover and protect this tissue (Asikainen et al., 2012; Ukkonen et al., 2017). Without the proper protective barrier, this leads to a diminished defence against mechanical and chemical substances, increasing risk of inflammation, erosion and ultimately chances of oral mucositis onset (Dawes, 2008; H. L. Gibbins et al., 2014; Hannig et al., 2017).

Furthermore, it has been established that when mucin 5B levels are affected due to external stimuli, there are also concurrent changes to secretion of α-amylase and other proteins (Ligtenberg and Almståhl, 2015), which corresponds with the current analysis of comparisons with oral mucositis and the protein composition on an individual basis that revealed positive and significant associations with a significantly reduced α-amylase unit concentration, α-amylase secretion rates, cystatin S concentration, and cystatin S secretion rate; all at time point 1.

With the co-operation of cystatin S and α-amylase in forming the mucosal pellicle being well established (Bradway et al., 1992; Ployon et al., 2016; Hannig et al., 2017), as well as cystatin S acting as a potential reaction agent with bacteria (Isemura et al., 1984) alongside α-amylase playing an important role in bacterial colonisation on oral surfaces by selectively maintaining the normal flora providing nutrients (Borghi et al., 2017; Cardoso et al., 2017), it stands to reason that their altered presence could be a reason for the oral mucositis severity determined by the reduced concentration and secretion rate of these aforementioned proteins, as further demonstrated by their significant associations with mucositis at Time Point 1. The cause of this being that altering both α-amylase and cystatin S could reduce microbial protection and increase the risk for secondary infections and pathogen bacterial colonisation, contributing to the mucositis inflammatory process (Sonis, 2011; Vanhoecke et al., 2015; Vasconcelos et al., 2016; Maria et al., 2017; Lynge Pedersen and Belstrøm, 2019). This microbial unbalance could be further affected by the altered salivary viscosity, related to both mucins' increased concentration, which impairs clearance of dietary carbohydrates and microorganisms from the oral cavity, in addition mucin 7 is capable of impeding the adherence of bacteria to oral surfaces being able to aggregate oral bacteria resulting in an efficient clearance by swallowing (Lynge Pedersen and Belstrøm, 2019).

Further analysis of comparisons of the protein composition on an individual basis revealed that albumin concentration and secretion rates were both positive associated with oral mucositis at T1. A possible explanation for this could be the findings from Chapter 3 that demonstrated albumin concentration was significantly elevated at T1, whilst secretion rate remained stagnant. This would go hand-in-hand with the significant association of this mentioned albumin with oral mucositis at this point (T1) , as during cases of mucositis**,** the oral mucosa will remain fragile, leading to plasma leakage and thus increasing this protein concentration in saliva (Almståhl et al., 2001; Eliasson et al., 2005).

In all the aforementioned cases, there appeared to be a distinct lack of statistical significance at T2. This could be potentially explained by the findings in Chapter 2 – whereby the low incidence rate of mucositis at time point 2 (mucositis presence was 9%) does not allow for any statistically significant associations to be formed. The cause of the low incidence rate of mucositis at T2 being the recovery of patients, which is to be expected as based on past literature showing recovery of oral health status within 1 to 2 years (Schuurhuis et al., 2011; Walker et al., 2011).

Immunoglobulin A was the one unique molecule observed that went against this trend, by presenting a statistically significant and positive association between IgA concentration and oral mucositis at both time Points 1 and 2, with IgA secretion rate also positive and significantly associated at Time Point 1, with a borderline association to oral mucositis at T2.

However, the cause of Immunoglobulin A's nonconformism to remain significant at T2 can be explained by the fact that, as shown in Chapter 3, concentration levels of IgA also did not alter at any time point following IMRT, in line with past studies that observed IgA concentrations in patients remain stable (Eliasson et al., 2005; Richards et al., 2017). One potential theory for their stability, or even potential increase during recovery (Martinez et al., 2007), is that IgA has been linked to mucosal pathologies, playing a role in mucosal defence (Engström and Engström, 1998; Joel B. Epstein et al., 2002; Hooper et al., 2012). Therefore, it may be linked to the recovery from oral mucositis rather than the onset of it. Furthermore, as Chapter 3 revealed that minor salivary gland functions are potentially spared from IMRT or recovered functionally, and previous studies have shown that minor buccal glands secrete IgA (Eliasson et al., 2006; de Paula et al., 2017), this could be the main origin of IgA that is allowing its levels to remain stable and facilitate these changes.

The presented correlative clinical and biochemical data provide insights in to the profiles of patients following head and neck cancer intensity-modulated radiation therapy and allow determination of potential cases that can serve as a reference for monitoring response to therapy. However, the utility of such associations for acting as predictive or diagnostic biomarkers is limited, therefore it is important to further utilise the data points to establish a model that could be used as a comprehensive analysis point to create diagnostic biomarkers, using a Receiver Operating Characteristic curve, or ROC curve. ROC curves are used to plot the diagnostic ability of a binary classifier in cases where discrimination thresholds are varied (in this case being the ability to accurately predict oral mucositis in instances where salivary protein thresholds vary).

It is important to note that a handful of past studies have utilised predictive salivary biomarkers to act as diagnostic tools for oral diseases (Miller et al., 2006; Gualtero and Suarez Castillo, 2016), and some have even evaluated the therapeutic opportunities of removing / substituting proteins (albeit inflammatory cytokines or chemokines, rather than regularly identifiable salivary proteins) in head and neck cancer patients (Elashoff et al., 2012; Sultani et al., 2012). However, as of yet, there are no studies to the author's knowledge, that have evaluated the possible associations among salivary protein variations in head and neck cancer patients and oral mucositis assessments. Oral mucositis research has been mainly focused on mucositis prevalence and severity, whether that be regarding quality of life of patients, patient suffering and consequences in cancer treatment alteration affecting its results regarding survival rates and relapse (Sonis, 2004a; Maria et al., 2017), or focused on therapeutic agents, studying effects of mitigation, delaying onset or limiting severity(Nomura et al., 2013; Villa and Sonis, 2016).

The current study proposed the use of novel salivary protein biomarkers for the early diagnosis of oral mucositis in head and neck cancer patients based on the analysis of ROC curves. Furthermore, this predictive model allows for identifying the potential severity of oncoming mucositis. The importance of the ability to predict and define a group of patients that might be in risk of developing a severe mucositis before IMRT would allow healthcare professionals to detect sufficiently early, to make decisions and plan an early intervention that must be effective to improve their quality of life, to minimize harm and reducing the economic burden related to additional care of palliate patient suffering (Park et al., 2004).

When using ROC diagnostic models, the performance of the model should be judged in the context of the analytic situation to which the test is applied. In this study, a salivary protein's cut-off point was chosen with a high sensitivity and a specificity over 50% for each protein analysed. In regard to AUC, every protein presented over 60% of this factor in concordance with the reports of Bradley (1997).

To date, no singular predictive model has been presented for accurately predicting the risk of developing and grade of severity in oral mucositis before radiotherapy. Therefore, this novel clinical model that analyses unstimulated whole mouth saliva by using quantitative experimental methods to detect certain proteins concentration and secretion rate pre-treatment (T0) in head and neck cancer patients to be compared with cut off points chosen from the ROC curve of the predictive model. The specific proteins concentration and secretion rate cut off points were selected with regards to their sensitivity and specificity to target this condition and classify patients in the clinic.

In this context, it was found that α-amylase with a cut-off point ≥30.14 units/ml had a rate of 93.1% correctly identify whether a patient would get mucositis with a high sensitivity rate of 92.59% and a specificity rate of 100%. Which is desirable in order to predict the possibility of experiencing an event if his/her estimated risk is above this given threshold. In addition, the area under the curve which integrates the curve over all cut off was AUC = 92.5%, indicating the best discriminatory power for that protein to differentiate between patients that present and not present the side effect, which is why it was selected.

Mucin 5B, both in terms of concentration and secretion, was also found to be an accurate predictor of severity of mucositis. More specifically, the ROC curves analysed mucin 5B concentration at Time Point 0 in order to predict the severity of the upcoming mucositis, with a cut-off point >16.16 μg/ml, it could predict with a 92.31% rate of correct classification for mucositis grades 1 & 2 (according to The WHO oral mucositis scale) with a sensitivity rate of 100%, specificity rate of 50%, and an area under the curve of 90.91%. Mucin 5B secretion rate with a cut-off point >4.65 μg/min could correctly classify 92.31% of mucositis grades 1&2 with a sensitivity rate of 100%, specificity rate of 50%, and an area under the curve of 86.36%.

Analysis to find an accurate predictor for oral mucositis severity grade 3, revealed that total protein concentration could be potentially used as a biomarker at Time Point 0 for upcoming

oral mucositis, with a correct classification rate of 62.5% when the cut-off point is >1.94 mg/ml, with a sensitivity rate of 90%, specificity rate of 50% and an area under the curve of 62.27%. It should be noted that this study could not be used to predict severity grade 4 of oral mucositis as none of the participants of this study manifested severity grade 4; however, it stands to reason that the study could be recreated with patients in the life-threatening condition of grade 4 and a biomarker found for the most severe grade of oral mucositis.

Whilst all the aforementioned analytical models identified potential onset and severity of oral mucositis during IMRT, ROC curves were also used to analyse salivary proteins collected at T0 as a possible molecular predictor of mucositis severity (grades 1&2) two weeks after IMRT and found that α-amylase with a cut-off point > 22.77 units/ml could accurately predict these severities with a correctly classified rate of 87.5%, a sensitivity of 100% and specificity of 50%, and an area under the curve of 91.67%.

The data found here demonstrates that whilst no single parameter could be utilised to fully elucidate the possibility of occurrence and severity of oral mucositis, the potential exists for combinations of certain protein concentrations and secretion rate signatures, more specifically total protein concentration, α-amylase concentration, mucin 5B concentration and mucin 5B secretion rate; to be utilised as a suitable suite for the early detection of oral mucositis prior to IMRT in a routine clinical situation. This possibility of finding a non-invasive and cost effective diagnostic method that can discriminate between patients with a high accuracy presents a crucial new discovery in the fight to reduce the serious side effects of head and neck cancer treatments, allowing for the reduction of risks for severe mucositis clinical outcomes and freeing up of extra resources for patient aftercare (Raber-Durlacher et al., 2013; Normando et al., 2017).

This goes against the grain of past studies, which did not focus on diagnostic biomarkers for severity or onset of this highly prevalent cancer treatment side effect, with the main bulk of research dedicated to efforts of reducing intensity and duration (Sonis, 2011; Epstein et al., 2012; Duarte et al., 2014; Franco et al., 2017; Normando et al., 2017), as symptoms still appear over 8 weeks after completion of treatment (Bensinger et al., 2008)and duration of oral mucositis in relation to radiotherapy can range from 3 to 12 weeks (Rosenthal and Trotti, 2009). The current model can predict mucositis prior to IMRT, as well as the potential severity,

which can allow for an increased range of treatments for achieving effective clinical results (Charalambous et al., 2018).

For such a condition with high prevalence in head and neck cancer patients, as well as the wide variety of further symptoms that affect sufferers of oral mucositis across the most severe cases (Sonis, 2011; Epstein et al., 2012), it becomes important to further validate these diagnostic models. The theoretical validation of the novel predictive method developed in this study was based on statistical procedures to determine the possibility of developing mucositis and severity predictor, on the basis of specific salivary proteins with this side effect at Time Points 0, 1 and 2. However, this method will need further experimental validation in prospective clinical studies, whether they be with a new range of patients, with patients who also exhibit more severe cases of oral mucositis, or with patients of more diverse age ranges. This would further validate these findings, as the ability to predict the risk of onset, as well as the severity, of oral mucositis in head and neck cancer patients undergoing IMRT could be of supreme importance, because it would allow healthcare workers to identify patients who are more at-risk, providing them with tailored oral care education along with development of appropriate preventive and supportive care for this high-risk group in order to keep them in the lowest severity, ultimately reducing pain and discomfort.

Such preventive measures would begin to take place prior to IMRT, by allowing the oncology team to keep patients in lower grades of severity for mucositis, reducing the probability of hospitalization, improving treatment outcome and quality of life. Clinical methods would include not only educational measures for maintaining oral health, but also detailed personalised care plans to reduce risk factors such as controlling oral infections in order to maintain bacterial homeostasis, dental and periodontal health, oral hygiene and nutritional intake monitoring. This has been demonstrated previously, with a study of paediatric cancer patients revealing that the degree of mucositis and pain levels diminished when children and their parents were educated regarding mouth care before chemotherapy(Yavuz and Bal Yılmaz, 2015). A further study concluded that providing a saline mouth rinse regimen and education programme on radiation-induced oral mucositis patients increases their social-emotional quality of life, in comparison to standard treatments for head and neck cancer patients(Huang et al., 2018).

Therefore, it is important to identify at-risk groups of patients prior to radiotherapy, in order for them to start with care actions, monitoring and even education programs for parents or next of kin; in an attempt to reduce the severity and risk of onset for oral mucositis (Yavuz and Bal Yılmaz, 2015; Huang et al., 2018).

All these measures may promote better physical and social-emotional quality of life, which is deeply impacted by oral mucositis symptoms, for example palliative measures for xerostomia such as no sugar chewing gums or mouth washes. This would all be done with the main aim of not only increasing patients' well-being but also increasing patient compliance with cancer treatment. Lack of compliance can typically lead to treatment interruptions, delays, altered dosages and fractioning due to regular visits to the emergency room and hospitalization to manage debilitating oral pain, receiving parenteral feeding and secondary infection treatment. This is of particular importance, not only because cancer treatment interruptions will threaten survival rate and remission (Scorsetti et al., 2010) but also because one of the key aspects behind oral mucositis is the multifactorial origin and pathobiology nature of this condition.

The multifactorial origin of oral mucositis is also one of the biggest drawbacks in a study to develop risk prediction models, with specific patient factors prevalent in this cohort such as smoking history and alcohol intake, both of which have been shown to dramatically affect immunological profiles and microvascular changes resulting in altered inflammatory responses (Al-Dasooqi et al., 2013). Oral mucositis has been defined as a complex cascade of biological events that includes microvascular damage, inflammatory and extracellular matrix alterations along with host microbiome interactions (Al-Dasooqi et al., 2013).

Another limiting factor of this study is that in order to establish a truly recognised predictive model – one that serves as a diagnostic tool to corroborate the direct relation with the condition and specific salivary proteins - it would require extensive validation with collaborative efforts from healthcare professionals. Currently, such predictions are made '*off the cuff*' based on each particular clinician's past experiences. Furthermore, oral screening and physical examinations in this group of patients were not easy to obtain before and after IMRT, due to the number of appointments that they undergo before cancer therapy as well as the multitude of examinations to fulfil cancer treatment requirements which may

complicate the possibility of adding extra bodily fluid collection tests to the regular appointments.

However, there are also many advantages to take in to consideration, in comparison with existing methods of diagnostics. One such advantage is the use of saliva for testing specific proteins before radiotherapy, with sample collection being relatively simple, pain-free, stress-free and cost-effective in comparison to blood or serum tests, making such a screening method performed before radiotherapy suitable to be validated in large clinical studies. Another advantage of the methods of this study is that the proteins utilised are salivary components related to lubrication and protective functions, therefore they could be collected with relative ease in order to identify other oral injuries such as xerostomia or taste palate changes, alongside the possibility to predict oral mucositis at the time of examination which can be reported using the same scale during radiotherapy.

This novel method of diagnosis that measures quantitatively specific salivary proteins in head and neck cancer patients before IMRT can make an important contribution in developing preventive therapeutic measures to diminish the impact of IMRT on quality of life of these cancer survivors along with improving treatment compliance.

Hence, through these findings we developed a working predictive model to use for future clinical applications and thus rejected our null hypothesis that there are no longitudinal associations between IMRT-induced changes in salivary markers with oral mucositis severity. We reason that the most probable links with oral mucositis at T1 are total protein secretion rate, as well as concentration, mucin 5B secretion rate, mucin 7 secretion rate, α-amylase concentration, as well as secretion rate, Albumin concentration, and Albumin secretion rate. This is of note, because whilst it is known that there are compositional changes to saliva in cases of oral mucosal diseases (Hassona and Scully, 2016), these associations have not been explored previous to this study and thus this was the first report of the combined application of salivary protein analysis and oral mucositis assessments, with the observations and clinical findings presenting potential implications in the pathophysiology of radiation-induced side effects.

Furthermore, links were identified at time Points 1 & 2 with both IgA secretion rate and concentration. However, these were dismissed as not likely links. The first set of findings were

then further analysed to reveal potential diagnostic biomarkers of oral mucositis, even prior to IMRT, in the analysis of total protein concentration, α-amylase concentration, mucin 5B concentration and mucin 5B secretion rate. These findings should be explored further in a wider study of participants, to validate the predictive models by assessing UWMS proteins collected before IMRT compare these outcomes with ROC curves cut-off points, classifying patients regarding their risk of presenting this side effect. Finally, comparing the predictive results with oral mucositis clinical onset and severity monitored by oncology team.

Such predictive models present a truly novel innovation in the preventative care of these groups of at-risk patients, making the potential personalised care plans and preventative measures a realistic possibility in order to modify risk factors. All of these preventive and educational care regimes would allow for patients to maintain a quality of life and social-emotional well-being similar to that of prior to treatment (Yavuz and Bal Yılmaz, 2015; Huang et al., 2018).

Chapter 5

5.1 Thesis Summary

Whenever new radiotherapy delivery treatments are introduced, they are evaluated both experimentally and clinically in relation to existing technologies, as well as in relation to further alternatives. However, an omission is that they are often not evaluated for their ability to partner with existing protocols to treat the well-documented side-effects of such treatments (Davidson et al., 2011; Heijkoop et al., 2014).

This study quantitatively evaluated Intensity-Modulated Radiation Therapy (IMRT) for their purported ability to spare non-cancer tissues in head & neck cancer therapy. More specifically, to assess salivary gland function, using non-invasive analysis, as traditional radiotherapy treatments produce a range of disruptive side effects due to the proximity of the tumour to the salivary glands (Tschoppe et al., 2010; Nutting et al., 2011).

Whilst previous studies have observed the changes involved following IMRT with the biochemical composition of saliva (Richards et al., 2017), xerostomia (Nutting et al., 2011), salivary flow rate (Hawkins et al., 2018), altered taste perception (Sio et al., 2016) and oral mucositis (Kouloulias et al., 2013), there still remain many unanswered questions, such as the long-term nature of these changes, as well as whether there are any associations between them. This study not only observed all the changes mentioned above, but also brought into scrutiny the clinical dental assessments, clinical observations and associations between all the aforementioned factors. In particular, analysing each factor and salivary protein composition, allowed identification of salivary biomarkers and their corresponding link to oral mucositis presence and severity.

The aim of this study was to analyse and evaluate the medium-term nature of oral side effects and changes in nine salivary protein composition, as well as their possible associations, in order to develop a clinical and laboratory-based model, built on these connections, for the prediction of the incidence and severity of oral mucositis in particular as it is one of the most frequent and more severe side effect of radiotherapy for head and neck cancer patients (Sonis, 1998, 2004a). Often defined as the dose-limiting side effect of RT, oral mucositis impairs daily activities such as the ability to swallow and feed normally, consequently

necessitating parenteral feeding, systemic analgesics, including opioids for pain control (Elting et al., 2008; Villa and Sonis, 2016).

The findings of this study revealed that despite IMRT's capacity to spare the parotid glands during treatment, it still provoked some persistent changes in UWMS flow rate, protein concentration and secretion rate, along with the appearance of clinical oral side effects related to radiotherapy that were seen longitudinally until one year post cancer treatment. This study also addressed the longitudinal associations of sialometry and protein composition of saliva on clinical symptoms, xerostomia and taste alteration, along with signs and symptoms of carious lesion and oral mucositis onset and severity.

These findings rejected the original the null hypothesis of the study – that ionising radiation has no effect on UWMS saliva composition at both time-points following IMRT. Overall, it was revealed that all side effects of radiotherapy were interconnected using statistical modelling of longitudinally processed data. This demonstrated that there were associations among a group of clinical symptoms, as well as clinical and biochemical salivary composition variation, that will affect the patient's quality of life, such as salivary gland function - which was observed clinically as a significant reduction in salivary flow rate resulting in xerostomia and altering taste perception. Additionally, a decreased salivary volume was accompanied and significantly associated with an altered protein composition/secretion rate, in addition it was reported the significant relationship between both and oral mucositis onset and severity. The associations found between reduced salivary volume and these clinical side effects are in line with past studies that observed saliva as a critical element in maintaining oral homeostasis and host protection (Hannig et al., 2017; Pitts et al., 2017).

The results of chapter 2 revealed that the clinical, and quality of life, side effects of IMRT, such as xerostomia, number of teeth, taste perception, were all in line with the existing radiotherapy literature (Almståhl et al., 2001; Dijkema et al., 2012; Laheij et al., 2015; Richards et al., 2017). These observations of significantly reduced salivary flow rate, which is a vital factor in maintaining oral health and functionality (Dawes et al., 2015), are backed up by the principle that an alteration of optimal properties of saliva will lead to a disequilibrium, increasing oral disease risk (Pitts et al., 2017).

Therefore, the next step was to analyse the biochemical properties of the saliva, with salivary proteins and mucins selected to be observed.

It was demonstrated that the total protein secretion rate was significantly reduced after IMRT, along with a reduced salivary volume. These changes were long lasting, without reaching baseline levels even after 1year post cancer treatment. However, total protein concentration significantly reduced at T1 showed a significant recovery at T2. Additionally, this study observed the influence and dependence of flow rate and protein concentration and secretion rate being significantly associated post IMRT.

The individual salivary proteins to be studied were selected according to their functions regarding oral homeostasis and their importance of maintaining a harmonious mutually beneficial relationship with microorganism, along with facilitating daily functions and directly or indirectly influencing the risk of developing oral diseases(Carpenter, 2013b; Proctor, 2016; Lynge Pedersen and Belstrøm, 2019).

Mucins were selected due to their ability to alter the wetting capacity and lubrication of mucosal surfaces (Vissink et al., 2010), with mucosal integrity being vital in HNC patients, due to the high rate of oral mucositis onset during radiotherapy along with xerostomia. Mucin 5B and 7 are vital molecules regarding mucosal pellicle to maintain a protective barrier ,hydration and bacterial adhesion (Hannig et al., 2017; Lynge Pedersen and Belstrøm, 2019). Mucins are capable of forming complex with IgA which is the main antibody in saliva and plays an important role in bacterial colonization (Dawes et al., 2015; Hemadi et al., 2017). If secretion of these glycoproteins in a functional state is not preserved, then it is unlikely further improvements in oral mucosa integrity and xerostomia are to be made.

α-Amylase was chosen to be observed as it is one of the most prevalent enzymes in saliva and is generally considered to be a reliable marker of serous cell function (therefore acting as a marker for parotid gland function (Proctor, 2016), along with its influence in oral biofilm formation and bacterial colonization.

Changes in concentrations of albumin have been linked to inflammatory processes and increased vascular permeability (Jensen et al., 2003), whilst cystatin S may be considered a biomarker in assessing submandibular glands function (Martini et al., 2017). PRPs can also act as biomarkers for submandibular and parotid glands, as they are secreted by parotid and submandibular glands and contain between 25-42% of proline amino-acids (Carpenter, 2013b).

Carbonic anhydrase VI plays a fundamental role in controlling taste sensation and taste bud growth, by facilitating the interaction between food particles and taste buds (Hunter, 2013).

Statherin was selected as it is secreted by all 3 major glands, being a phosphoprotein secreted from parotid, submandibular and sublingual glands. Furthermore, past studies have also observed that albumin, statherin, cystatin S, PRP, mucins 5b and 7 form acquired enamel pellicle, reducing the possibility of erosion and abrasion, determining biofilm formation, keeping mineral equilibrium by maintaining a high concentration of calcium and buffer capacity (H. L. Gibbins et al., 2014; Hannig et al., 2017). Therefore, all of these salivary bio-composites were observed, as it is important to assess concentration and secretion rate of these salivary proteins in order to evaluate overall functionality (Dawes et al., 2015).

Overall, the variation of biochemical composition of UWM saliva in HNC patients undergoing radiotherapy were in line with other authors outcomes (Dijkema et al., 2012; Richards et al., 2017). However, this study added more results, including secretion rate of 9 proteins and a longer follow-up period with a baseline outcome to compare. Moreover, there was analysed the associations between these proteins regarding its functions with clinical oral side effects reported in previous chapter.

The outcomes revealed that there were significant protein concentration and secretion rate variations in saliva as a side effect of the cancer treatment. These findings altered normal feelings in each individual's mouth and led to an increased risk of oral diseases regarding oral tissues (Chaudhury et al., 2015; Frenkel and Ribbeck, 2015; Vijay et al., 2015; Hemadi et al., 2017). This was demonstrated with the significant associations between the 9 proteins analysed and dry mouth feeling, taste acuity, as well as salivary flow rate as reported in chapter 3. Such number of proteins longitudinal association analysis have not been performed in the literature. Regarding mucin 5B associations with salivary flow rate are similar to past findings, such as the Dijkema research group reporting trends of xerostomia after radiotherapy with only protein, mucin 5b levels, in submandibular saliva from patients (Dijkema et al., 2012).

These side effects were observed in this particular study mostly in the form of oral mucositis, a morbid condition that occurred in 77% of this study's participants, as shown in Chapter 2**,** with 45% of those exhibiting Grade 2 mucositis and 51% exhibiting Grade 3 mucositis. These grades were recorded according to the WHO oral mucositis scale, a grading system that documents the severity and prevalence of mucositis, with the scale being one of the most commonly used in order to track this condition. However, there is no universal scale for measuring mucositis, with this being one of the biggest drawbacks in oral mucositis research.

Other scales used by others include the Radiation Therapy Oncology Group scale (RTOG) and European Organisation for Research and Treatment of Cancer scale (EORTC). Whilst RTOG / EORTC are focused on patients' levels of pain and suffering, in order to measure the necessity of analgesia; the WHO oral mucositis scale is focused on patients' functionality in relation to the capacity to eat solid food (Sciubba and Goldenberg, 2006; Sroussi et al., 2017). However, these are all subjective measurements, with evaluative outcomes dependent on a physician's clinical experience and training in order to clearly define categories on each relevant scale. This presents a clear limitation of all studies observing oral mucositis as it makes it more arduous to perform validation studies because these instruments are vulnerable to misinterpretation and clinical subjectivity.

Furthermore, questionnaire-based points of data collection, such as surveying for "dry mouth sensations" in order to determine xerostomia, are another limiting factor for this study as the survey's respondents are not clinical experts. This leads to subjectivity, misinterpretations and omission errors during data collection, whether that be due to two subjects having different subjective value points or whether a subject might submit rushed/false data to speed up their experience as a result of the length of the surveys. Thus, these measurement scales require adaptation for patient use, as almost all scales are designed for physician use (Ho et al., 2010). This has been demonstrated in past literature that reported differences between patient-reported data and clinically observed data (Bentzen, 1998; Davidson et al., 2007). Despite the aforementioned limitations, the methodology used in this instance for determining potential xerostomia is one that has been long established and used by others for decades (Fox et al., 1987). The questionnaire used for taste variation is an extract from the Late Effects on Normal Tissues (LENT), Subjective, Objective, Management, Analytic (SOMA) survey (Davidson et al., 2007; Ho et al., 2010). The use of LENT-SOMA is, once again similar to our xerostomia methodology, well established and has even been included into the National Cancer Institute's (NCI) documented methodologies (Davidson et al., 2007; Ho et al., 2010).

The lack of uniformity in measurement scales for such clinical oral studies could be one limiting factor affecting long term comparative reviews, which is of particular importance due to the fact that, at the present time, there are no effective, direct treatments to reduce oral mucositis onset, severity and duration. Also, there are no medications to prevent oral mucositis, even though there are numerous studies testing different products, promoting oral hygiene, food advice, reducing alcohol intake and quitting smoking, these are only palliative

measures (Park et al., 2004). However, more recently, there have been studies focussed on the use of antibodies in IMRT patients to neutralize the inflammatory response of mucositis, as well as increasing defensive response (Villa and Sonis, 2016; Normando et al., 2017).

Another important factor in this study was the enrolment of patients with several tumour locations and IMRT treatment sites. It was revealed that despite the fact that each individual's tumour location was different, IMRT's capacity to spare the glands, and therefore the salivary protein profile and secretion rates, were still affected in all patients, along with development of clinical side effects. The primary tumour sites were mostly tonsillar (*n=12*), oropharynx (*n=6*) and tongue (*n=5*), which are closely proximate, affecting more than 50% of the examined group, with the minority divided into five different subsites. Another study that supports the current findings that different IMRT treatment sites had no effect on consequential side effects, is a demonstration of how different primary tumour sites are unlikely to affect the xerostomia prevalence when the radiation-dose parameters are matched between the experimental groups (Richards, 2014).

Furthermore, the current study's radiation dosage and fractioning regime were delivered in a range that was quite narrow, varying between 55-65 Gy with an average fractioning of 2Gy per day. This reduced the probability of tumour sites affecting the prevalence of side effects, as backed up by a previous study that observed how site specific post-radiotherapy treatments were of no difference when the radiation dosage to the salivary gland remained below a certain level (Pringle et al., 2013) this could be is explained by the bystander effect, which consider the Reactive Oxygen Species (ROS) as the possible source of the signal and in the propagation of radiation effects from irradiated cells to adjacent unirradiated cells (Mothersill et al., 2019).

This is similar to our findings of oral mucositis, whereby it was observed that there were no differences in onset or grading, in contrast to prior literature that stated that the risk of this side effect varies with the site of the primary tumour, (Chao et al., 2001; Pinna et al., 2015; Strojan et al., 2017; Sroussi et al., 2017).

Chapter 4 focussed on establishing whether a clear predictive biomarker for mucositis can be identified prior to the commencement of radiotherapy treatment. Once such potential biomarkers were identified, their specificity and sensitivity rate of predictive accuracy were

analysed, then a further step was taken to analyse each grading of mucositis against the diagnostic ability of predictions using a Receiver Operating Characteristic (ROC) curve.

The findings revealed that α-amylase concentration, mucin 5b concentration and secretion rate, as well as total protein concentration, can all be suitable as biomarkers for the detection of oral mucositis, prior to IMRT by collecting UWMS. Such predictive biomarkers present new possibilities in oral mucositis research and represent an opportunity for clinical trials with this novel method allowing for speeding up research and routine laboratory techniques, as they were found to be reliable, as well as allowing for non-invasive sample collection and cost-effective diagnostic methods during a routine clinical appointment. This represents not only an opportunity to create a clear identifier to prevent cancer treatment interruption (Turner et al., 2013) but also the opportunity to create personalised preventive care regimes in order to reduce the effects on mucositis, prior to even the development of oral mucositis symptoms (Normando et al., 2017).

However, such conclusions should be observed further and further evaluated, as salivary components display redundancy of function, each often having more than one (Dowd, 1999). This suggests that the findings of Chapter 4, whilst important as a base point to begin further evaluations to find biomarkers for oral mucositis prior to IMRT taking place, could also be affecting other functions or indicating other protein pathway networks in place that are acting on these oral functions. Therefore, it is important that such predictive models are extensively tested and validated in a longitudinal prospective randomised clinical trial including more patients, perhaps multicentre, maintaining the robustness and reproducibility of the saliva collection, storage and laboratory assays along with keeping the oral mucositis assessment and adding more clinical features to have a full characterization of this condition during and after IMRT (Elashoff et al., 2012).

The evaluation of salivary components revealed a relationship between oral mucositis and α-amylase concentration, mucin 5b concentration and secretion rate, as well as total protein concentration. However, it has not been able to articulate the reasoning behind these findings, as to the role of each salivary component and how they could be acting within the relationship with oral mucositis. The determination of the role of each biomarker would be an important part in the translation of these laboratory diagnostics into clinically applicable tests (Podzimek et al., 2016), but until each biomarker has been evaluated in longitudinal

studies across a large pool of patients, as well as evaluated for the causalities behind them, they are not considered truly clinically applicable markers (Podzimek et al., 2016).

Once such evaluations are concluded, the next steps would be for the development of a protocol to guide clinical diagnosis, deliver accurate prognostic information and monitor disease progression, allowing for dentists and clinicians to develop not only pre-emptive care plans but also educational programs tailored for each patient. Studies reveal that development of such personalised care programs based on individual diagnostics can help lower the severity of mucositis, potentially delay onset and progression in head and neck cancer patients, along with reducing a plethora of symptoms which combined effect can drastically reduce quality of life of these patients (Sonis, 2011; Epstein et al., 2012), as well as the ease for the patients' families (Yavuz and Bal Yılmaz, 2015; Huang et al., 2018).

In conclusion, whilst the patient's role in mitigating the toxic side effects of head and neck cancer therapy is increasingly acknowledged, allowing for the potential implementation of personalised educational and care plans that can drastically reduce quality of life of these patients (Dowd, 1999; Sonis, 2011), without the diagnostic ability of biomarkers it is nigh-on-impossible to determine which therapeutics are necessary ahead of treatment. Therefore, this study evaluated all aspects of saliva and the oral cavity's eco-system and determined not only functional insights into nine proteins and their relationship with clinical observations (such as taste perception changes), but also revealed that salivary α-amylase concentration, mucin 5B concentration and secretion rate, as well as total salivary protein concentration, can all be suitable on their individual basis to be used as molecular markers for the detection of oral mucositis, prior to IMRT by collecting UWM saliva. And whilst it has been known that there are compositional changes to saliva in cases of oral diseases (Hassona and Scully, 2016), this has been the first report of its kind to combine the application of salivary protein analysis and oral mucositis assessments, with the observations and clinical findings presenting potential implications in the pathophysiology of radiation-induced side effects, and even finding a biomarker that could correctly classify 92.31% of cases ahead of IMRT, which may have a huge potential to guide future pre-clinical studies and possibly therapeutic interventions.

5.2 Summary and Suggestions for Future Work

The outcomes of this thesis suggest the need for further clinical and biochemical investigations, regarding IMRT side effects in the oral cavity. Despite the advances in evaluations of side effects that were reported in the literature, it is evident that radiation-induced salivary gland hypofunction remains in sizable quantities of the patient group, which affects the oral cavity environment significantly.

1. Biochemically, from the data presented in this thesis the next steps regarding salivary composition variation post IMRT should be assessed mucin functionality. This is based on their structure composition: Analysis of the molecular composition of Mucins 5b and 7 from UWMS and assessing the glycosylation protein rate in order to analyse mucin hydration capacity – which is an important component in mucin functionality and structure (Chaudhury et al., 2015). Periodic acid–Schiff staining directly on protein gels is capable of detecting mucin glycosylation structure. Furthermore, to detect the protein core of the mucins, western blot analysis using antibodies is required. The relative proportion of glycosylated mucin to mucin protein (glycan/protein proportions), allowing the estimation of glycosylation per unit protein, can be calculated by dividing PAS staining– detected mucins with antibody-detected mucins.
2. Increase the length of follow-up periods of the clinical trial participants. This involves clinical oral assessment and biochemical analysis of saliva composition, adding time points up to 2.5 years. A singular follow up after just one year is insufficient to detect changes in teeth integrity regarding radiation caries onset and development, along with the possible associations with volume and biochemical (proteins) composition of saliva that are relevant protective/modulating factors that modify the caries process.
3. It would be useful to determine the exact dose of radiation received by each salivary gland, including the left and the right as well as the volume of the gland irradiated. This information can be obtained for each patient from the radiation treatment planning software, by manually contouring salivary glands using a 3D pencil beam. The delineation permits to obtain a three-dimensional dose distribution for each gland in each patient and the average of the mean and the maximum point doses for each

defined region. These dosimetric values allow for more accurate determinations of the influence of radiation on clinical outcomes and salivary proteins.

4. Design a new prospective clinical trial - including new patients and extra time points during and early after IMRT to the 3 investigated in this study.

   The data of this thesis highlights potential implications of oral mucositis regarding assessment and characterization during and after IMRT, along with the validation of possible markers for this side effect. Clinical evaluation will incorporate new factors to the WHO scale, such as anatomy, size of ulcers and report the regions affected throughout the oral cavity in order to have an accurate picture of this side effect. Clinical assessment along with UWMS sample collection before IMRT, during IMRT, 2 weeks post IMRT, 6 weeks post IMRT and 3, 6, 12 months. Biochemical analysis of proteins before IMRT in order to compare with cut off points obtained from the ROC curves, for each protein, classification of patients whether they are or not in risk of developing of oral mucositis as well as severity. Follow-up of these patients during and after the cancer treatment to corroborate our findings with the clinical outcomes, to validate the predictive model proposed in this thesis.

5. Assessing outcomes of xerostomia without the use of patient reported surveys.

   Despite the fact that IMRT is purported to spare salivary glands during treatment, this study showed a high prevalence of xerostomia reported by patients along with a significant reduction of flow rate and altered mucin 5B and 7 composition and secretion rate. It has been established that sublingual and minor salivary glands are the sole sources of Mucins 5B and 7 in the oral cavity (Carpenter et al., 2014; Proctor, 2016). If secretion of these glycoproteins in a functional state is not preserved, further improvements in dry mouth perception would be unlikely. Therefore, the outcomes of xerostomia, to assess the level of radiation side-effects on these salivary glands, should be assessed using one of the multiple objectives and analytical techniques, to assess oral-dryness and the functional integrity of saliva. One such method is assessing the extensional properties of saliva via Spinnbarkeit (stringiness) measurements using the Neva Meter (IMI-0501; Ishikawa Ironworks Co., Kobe, Japan).

6. Analysis of minor salivary glands via the new prospective clinical trial designs.

   Current data on minor salivary glands in head and neck cancer patients seem to be limited, thus it is imperative to analyse minor salivary glands flow rate and protein

concentration regarding mucosal pellicle. The prospective trial models should contain detailed assessments of quantitative and qualitative measures of mucin function and patient symptoms. Residual mucosal saliva collection from different locations in the mouth such as anterior hard palate, buccal and lower labial surfaces. This collection will utilise filter paper discs, Sialo Paper strips (Oraflow Inc., Smithtown, NY, USA) and measured using Periotron 8000 (Oraflow Inc). This would allow for analysis of cases whereby patients still report “dry mouth sensation” despite steady salivary flow rates, caused due to a depravation of surface mucosa of its salivary wetting characteristics (Ranc et al., 2006).

Bibliography

Al-Dasooqi, N. et al. (2013) Emerging evidence on the pathobiology of mucositis. Supportive Care in Cancer

Almståhl, A. et al. (2019) Explorative study on quality of life in relation to salivary secretion rate in head and neck cancer patients treated with radiotherapy up to 2 years post treatment. *International Journal of Dental Hygiene*. [Online]

Almståhl, A. et al. (2001) Lactoferrin, amylase and mucin MUC5B and their relation to the oral microflora in hyposalivation of different origins. *Oral Microbiology and Immunology*. [Online]

Andreassen, C. N. (2010) Editorial: Searching for genetic determinants of normal tissue radiosensitivity - Are we on the right track? Radiotherapy and Oncology

Andrews, N. & Griffiths, C. (2001) Dental complications of head and neck radiotherapy: Part 2. *Australian dental journal*.

Ann, D. K. et al. (1987) Induction of tissue-specific proline-rich protein multigene families in rat and mouse parotid glands by isoproterenol. Unusual strain differences of proline-rich protein mRNAs. *Journal of Biological Chemistry*.

Anon (1979) WHO handbook for reporting results of cancer treatment. [Online]

Aroonsang, W. et al. (2014) Influence of substratum hydrophobicity on salivary pellicles: organization or composition? *Biofouling*. [Online]

Asikainen, P. et al. (2015) Microplicae - Specialized surface structure of epithelial cells of wet-surfaced oral Mucosa. Ultrastructural Pathology

Asikainen, P. et al. (2012) The defence architecture of the superficial cells of the oral mucosa. *Medical Hypotheses*. [Online]

Asikainen, P. J. et al. (2017) Radiation-induced changes in the microstructure of epithelial cells of the oral mucosa: A comparative light and electron microscopic study. *Journal of Oral Pathology and Medicine*. [Online]

Atmeh, A. R. et al. (2015) Calcium silicate cement-induced remineralisation of totally

demineralised dentine in comparison with glass ionomer cement: Tetracycline labelling and two-photon fluorescence microscopy. *Journal of Microscopy*. [Online]

BANNISTER, A. J. et al. (1989) Basic proline-rich proteins of murine parotid glands: Induction of mRNA by isoprenaline and post-secretion processing. *European Journal of Biochemistry*. [Online]

Baron, A. et al. (2008) Functional aspects of the human salivary cystatins in the oral environment. *Oral Diseases*. [Online]

Baskar, R. et al. (2012) Cancer and radiation therapy: Current advances and future directions. International Journal of Medical Sciences

Belstrøm, D. et al. (2016) Bacterial composition in whole saliva from patients with severe hyposalivation - a case-control study. *Oral Diseases*. [Online]

Bensinger, W. et al. (2008) NCCN task force report: Prevention and management of mucositis in cancer care. JNCCN Journal of the National Comprehensive Cancer Network

Bentzen, S. M. (1998) Towards evidence based radiation oncology: Improving the design, analysis, and reporting of clinical outcome studies in radiotherapy. *Radiotherapy and Oncology*. [Online]

Borghi, G. N. et al. (2017) Relationship among α amylase and carbonic anhydrase VI in saliva, visible biofilm, and early childhood caries: a longitudinal study. *International Journal of Paediatric Dentistry*. [Online]

Bradley, A. P. (1997) The use of the area under the ROC curve in the evaluation of machine learning algorithms. *Pattern Recognition*. [Online]

Bradway, S. D. et al. (1992) Formation of salivary-mucosal pellicle: The role of transglutaminase. *Biochemical Journal*. [Online]

Brand, H. S. et al. (2014) Saliva and wound healing. *Monographs in Oral Science*. [Online]

Brandtzaeg, P. (2009) Mucosal immunity: Induction, dissemination, and effector functions. Scandinavian Journal of Immunology

Brandtzaeg, P. (2013) ‘Mucosal immunity in a healthy gut’, in *Diet, Immunity and Inflammation*. [Online]. p.

Breneman, J. C. (1994) Ionizing radiation therapy in dermatology. *Journal of the American Academy of Dermatology*. [Online]

Brennan, P. A. et al. (2017) Intensity-modulated radiotherapy in head and neck cancer — an update for oral and maxillofacial surgeons. British Journal of Oral and Maxillofacial Surgery

Bressan, V. et al. (2016) The effects of swallowing disorders, dysgeusia, oral mucositis and xerostomia on nutritional status, oral intake and weight loss in head and neck cancer patients: A systematic review. Cancer Treatment Reviews

Broadbent, J. M. & Thomson, W. M. (2005) For debate: Problems with the DMF index pertinent to dental caries data analysis. Community Dentistry and Oral Epidemiology

Buzalaf, M. A. R. et al. (2011) Mechanisms of action of fluoride for caries control. *Monographs in Oral Science*. [Online]

Cardoso, A. A. et al. (2017) Influence of salivary parameters in the caries development in orthodontic patients—an observational clinical study. *International Journal of Paediatric Dentistry*. [Online]

Carpenter, G. et al. (2014) Composition of enamel pellicle from dental erosion patients. *Caries Research*. [Online]

Carpenter, G. H. et al. (2000) Sialochemical markers of salivary gland involvement with Sjogren’s syndrome secondary to rheumatoid arthritis and primary biliary cirrhosis. *Journal of Oral Pathology and Medicine*. [Online]

Carpenter, G. H. (2013a) The Secretion, Components, and Properties of Saliva. *Annual Review of Food Science and Technology*. [Online] 4 (1), 267–276. [online]. Available from: http://www.annualreviews.org/doi/10.1146/annurev-food-030212-182700.

Carpenter, G. H. (2013b) The Secretion, Components, and Properties of Saliva. *Annual Review of Food Science and Technology*. [Online]

Castro, I. et al. (2013) Oral dryness in Sjögren's syndrome patients. Not just a question of water. Autoimmunity Reviews

Chao, K. S. C. et al. (2001) A prospective study of salivary function sparing in patients with head-and-neck cancers receiving intensity-modulated or three-dimensional radiation therapy: Initial results. *International Journal of Radiation Oncology Biology Physics*. [Online]

Charalambous, M. et al. (2018) The effect of the use of thyme honey in minimizing radiation - induced oral mucositis in head and neck cancer patients: A randomized controlled trial. *European Journal of Oncology Nursing*. [Online]

Chaudhury, N. M. A. et al. (2015) Changes in saliva rheological properties and mucin glycosylation in dry mouth. *Journal of Dental Research*. [Online]

Chaudhury, N. M. A. et al. (2016) Reduced mucin-7 (MUC7) sialylation and altered saliva rheology in Sjögren's syndrome associated oral dryness. *Molecular and Cellular Proteomics*. [Online]

Chen, J. L. Y. et al. (2017) Intensity-modulated radiation therapy achieves better local control compared to three-dimensional conformal radiation therapy for T4-stage nasopharyngeal carcinoma. *Oncotarget*. [Online]

Chi, A. C. et al. (2015) Oral cavity and oropharyngeal squamous cell carcinoma-an update. *CA: A Cancer Journal for Clinicians*. [Online]

Chiappin, S. et al. (2007) Saliva specimen: A new laboratory tool for diagnostic and basic investigation. Clinica Chimica Acta

Cone, R. A. (2009) Barrier properties of mucus. Advanced Drug Delivery Reviews

Crawford, J. et al. (1975) Minor salivary glands as a major source of secretory immunoglobin A in the human oral cavity. *Science*. [Online]

Davidson, M. T. M. et al. (2011) Assessing the role of volumetric modulated arc therapy (VMAT) relative to IMRT and helical tomotherapy in the management of localized, locally advanced, and post-operative prostate cancer. *International Journal of Radiation Oncology Biology Physics*. [Online]

Davidson, S. E. et al. (2007) Improving the Capture of Adverse Event Data in Clinical

Trials: The Role of the International Atomic Energy Agency. *International Journal of Radiation Oncology Biology Physics*. [Online]

Dawes, C. (2008) Salivary flow patterns and the health of hard and soft oral tissues. Journal of the American Dental Association

Dawes, C. et al. (2015) The functions of human saliva: A review sponsored by the World Workshop on Oral Medicine VI. Archives of Oral Biology

Dawes, C. & Ong, B. Y. (1973) Circadian rhythms in the flow rate and proportional contribution of parotid to whole saliva volume in man. *Archives of Oral Biology*. [Online] 18 (9), 1145–1153.

Deng, J. et al. (2015) Dental demineralization and caries in patients with head and neck cancer. Oral Oncology

Denny, P. et al. (2008) The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions. *Journal of Proteome Research*. [Online]

Diaz-Sanchez, R. M. et al. (2015) Double-blind, randomized pilot study of bioadhesive chlorhexidine gel in the prevention and treatment of mucositis induced by chemoradiotherapy of head and neck cancer. *Medicina Oral, Patologia Oral y Cirugia Bucal*. [Online]

Dijkema, T. et al. (2008) Large Cohort Dose-Volume Response Analysis of Parotid Gland Function After Radiotherapy: Intensity-Modulated Versus Conventional Radiotherapy. *International Journal of Radiation Oncology Biology Physics*. [Online]

Dijkema, T. et al. (2012) MUC5B levels in submandibular gland saliva of patients treated with radiotherapy for head-and-neck cancer: A pilot study. *Radiation Oncology*. [Online]

Dijkema, T. et al. (2010) Parotid gland function after radiotherapy: The combined Michigan and Utrecht experience. *International Journal of Radiation Oncology Biology Physics*. [Online]

Dodd, M. J. et al. (2003) Radiation-induced mucositis: A randomized clinical trial of micronized sucralfate versus salt & soda mouthwashes. *Cancer Investigation*.

[Online]

Doty, R. L. et al. (2017) Taste quality confusions: Influences of age, smoking, PTC taster status, and other subject characteristics. *Perception*. [Online]

Dowd, F. J. (1999) Saliva and dental caries. Dental clinics of North America

Duarte, V. M. et al. (2014) Comparison of dental health of patients with head and neck cancer receiving IMRT vs conventional radiation. *Otolaryngology - Head and Neck Surgery (United States)*. [Online]

Duke, R. L. et al. (2005) Dental status and quality of life in long-term head and neck cancer survivors. *Laryngoscope*. [Online]

Ebersole, J. L. et al. (2015) Targeted salivary biomarkers for discrimination of periodontal health and disease(s). *Frontiers in Cellular and Infection Microbiology*. [Online]

Eisbruch, A. et al. (1999) Dose, volume, and function relationships in parotid salivary glands following conformal and intensity-modulated irradiation of head and neck cancer. *International Journal of Radiation Oncology Biology Physics*. [Online]

Elashoff, D. et al. (2012) Prevalidation of salivary biomarkers for oral cancer detection. *Cancer Epidemiology Biomarkers and Prevention*. [Online]

Eliasson, L. et al. (2009) Feeling of dry mouth in relation to whole and minor gland saliva secretion rate. *Archives of Oral Biology*. [Online]

Eliasson, L. et al. (2005) Minor gland saliva flow rate and proteins in subjects with hyposalivation due to Sjögren's syndrome and radiation therapy. *Archives of Oral Biology*. [Online]

Eliasson, L. et al. (2006) Minor salivary gland secretion rates and immunoglobulin A in adults and the elderly. *European Journal of Oral Sciences*. [Online]

Eliasson, L. & Carlén, A. (2010) An update on minor salivary gland secretions. European Journal of Oral Sciences

Elting, L. S. et al. (2008) Patient-reported measurements of oral mucositis in head and neck cancer patients treated with radiotherapy with or without chemotherapy: Demonstration of increased frequency, severity, resistance to

palliation, and impact on quality of life. *Cancer*. [Online]

Englander, H. R. et al. (1959) The Effects of Saliva on the pH and Lactate Concentration in Dental Plaques:I. Caries-Rampant Individuals. *Journal of Dental Research*. [Online]

Engström, G. N. & Engström, P. E. (1998) Effects of tobacco smoking on salivary immunoglobulin levels in immunodeficiency. *European Journal of Oral Sciences*. [Online]

Epstein, J. B. et al. (2012) Oral complications of cancer and cancer therapy: From cancer treatment to survivorship. *CA Cancer Journal for Clinicians*. [Online]

Epstein, J. B. et al. (2002) Quality of life, taste, olfactory and oral function following high-dose chemotherapy and allogeneic hematopoietic cell transplantation. *Bone Marrow Transplantation*. [Online]

Epstein, J. B. et al. (2019) Taste disorders following cancer treatment: report of a case series. *Supportive Care in Cancer*. [Online]

Epstein, Joel B. et al. (2002) The role of salivary function in modulating chemotherapy-induced oropharyngeal mucositis: A review of the literature. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics*. [Online]

Epstein, J. B. et al. (2016) Understanding the impact of taste changes in oncology care. *Supportive Care in Cancer*. [Online]

Epstein, J. B. & Barasch, A. (2010) Taste disorders in cancer patients: Pathogenesis, and approach to assessment and management. Oral Oncology

Fábián, T. K. et al. (2015) Molecular mechanisms of taste recognition: Considerations about the role of saliva. International Journal of Molecular Sciences

Fábián, T. K. et al. (2012) Salivary defense proteins: Their network and role in innate and acquired oral immunity. International Journal of Molecular Sciences

Fan, G. (2007) Symptom Clusters in Cancer Patients: A Review of the Literature. *Current Oncology*. [Online]

Fejerskov, O. (2004) ‘Changing paradigms in concepts on dental caries: Consequences for oral health care’, in *Caries Research*. [Online]. 2004 p.

Fox, P. C. et al. (1987) Subjective reports of xerostomia and objective measures of salivary gland performance. *Journal of the American Dental Association (1939)*. [Online]

Franco, P. et al. (2017) Prospective assessment of oral mucositis and its impact on quality of life and patient-reported outcomes during radiotherapy for head and neck cancer. *Medical Oncology*. [Online]

Fränzel, W. & Gerlach, R. (2009) The irradiation action on human dental tissue by X-rays and electrons - a nanoindenter study. *Zeitschrift fur Medizinische Physik*. [Online]

Franzmann, E. J. et al. (2012) Salivary protein and solCD44 levels as a potential screening tool for early detection of head and neck squamous cell carcinoma. *Head and Neck*. [Online]

Frasseto, F. et al. (2012) Relationship among salivary carbonic anhydrase vi activity and flow rate, biofilm ph and caries in primary dentition. *Caries Research*. [Online]

Frenkel, E. S. & Ribbeck, K. (2015) Salivary mucins in host defense and disease prevention. *Journal of Oral Microbiology*. [Online]

Funegård, U. et al. (1994) Parotid saliva composition during and after irradiation of head and neck cancer. *European Journal of Cancer. Part B: Oral Oncology*. [Online]

Gabryel-Porowska, H. et al. (2014) Mucin levels in saliva of adolescents with dental caries. *Medical Science Monitor*. [Online]

Gao, L. et al. (2015) Exploring the variation of oral microbiota in supragingival plaque during and after head-and-neck radiotherapy using pyrosequencing. *Archives of Oral Biology*. [Online]

Gao, X. et al. (2016) Salivary biomarkers for dental caries. Periodontology 2000

GB, S. et al. (2015) Effect of low-level laser therapy on inflammatory mediator

release during chemotherapy-induced oral mucositis: a randomized preliminary study. Lasers in medical science

Van Gestel, D. et al. (2011) Intensity-modulated radiotherapy in patients with head and neck cancer: A European single-centre experience. *British Journal of Radiology*. [Online]

Ghosh-Laskar, S. et al. (2016) 'Prospective randomized controlled trial to compare 3-dimensional conformal radiotherapy to intensity-modulated radiotherapy in head and neck squamous cell carcinoma: Long-term results', in *Head and Neck*. [Online]. 2016 p.

Gibbins, H. L. et al. (2014) Concentration of salivary protective proteins within the bound oral mucosal pellicle. *Oral Diseases*. [Online]

Gibbins, Hannah L. et al. (2014) What interactions drive the salivary mucosal pellicle formation? *Colloids and Surfaces B: Biointerfaces*. [Online]

Gil-Montoya, J. A. et al. (2008) Evaluation of the clinical efficacy of a mouthwash and oral gel containing the antimicrobial proteins lactoperoxidase, lysozyme and lactoferrin in elderly patients with dry mouth - A pilot study. *Gerodontology*. [Online]

Gil-Montoya, J. A. et al. (2016) Treatment of xerostomia and hyposalivation in the elderly: A systematic review. Medicina Oral Patologia Oral y Cirugia Bucal

Gomes-Silva, W. et al. (2017) Postradiation Matrix Metalloproteinase-20 Expression and Its Impact on Dental Micromorphology and Radiation-Related Caries. *Caries Research*. [Online]

Gonçalves, L. M. N. et al. (2014) Radiation therapy alters microhardness and microstructure of enamel and dentin of permanent human teeth. *Journal of Dentistry*. [Online]

Gorr, S. U. (2009) Antimicrobial peptides of the oral cavity. *Periodontology 2000*. [Online]

Granger, D. A. et al. (2007) Integration of salivary biomarkers into developmental and behaviorally-oriented research: Problems and solutions for collecting specimens. *Physiology and Behavior*. [Online]

Gualtero, D. F. & Suarez Castillo, A. (2016) Biomarkers in saliva for the detection of oral squamous cell carcinoma and their potential use for early diagnosis: A systematic review. Acta Odontologica Scandinavica

Hahn Berg, I. C. et al. (2004) Intraoral lubrication of PRP-1, statherin and mucin as studied by AFM. *Biofouling*. [Online]

Hancock, P. J. et al. (2003) Oral and dental management related to radiation therapy for head and neck cancer. Journal (Canadian Dental Association)

Hannig, C. et al. (2017) The mucosal pellicle – An underestimated factor in oral physiology. Archives of Oral Biology

Hannig, M. et al. (2006) Does irradiation affect the protein composition of saliva? *Clinical Oral Investigations*. [Online]

Hassona, Y. & Scully, C. (2016) Salivary changes in oral mucosal diseases. Periodontology 2000

Hawkins, P. G. et al. (2018) Sparing all salivary glands with IMRT for head and neck cancer: Longitudinal study of patient-reported xerostomia and head-and-neck quality of life. *Radiotherapy and Oncology*. [Online]

Heijkoop, S. T. et al. (2014) Clinical implementation of an online adaptive plan-of-the-day protocol for nonrigid motion management in locally advanced cervical cancer IMRT. *International Journal of Radiation Oncology Biology Physics*. [Online]

Helenius, L. M. J. et al. (2005) Oral and salivary parameters in patients with rheumatic diseases. *Acta Odontologica Scandinavica*. [Online]

Hemadi, A. S. et al. (2017) Salivary proteins and microbiota as biomarkers for early childhood caries risk assessment. International journal of oral science

Ho, K. F. et al. (2010) Comparison of patient-reported late treatment toxicity (LENT-SOMA) with quality of life (EORTC QLQ-C30 and QLQ-H&N35) assessment after head and neck radiotherapy. *Radiotherapy and Oncology*. [Online]

Hooper, L. V. et al. (2012) Interactions between the microbiota and the immune system. Science

Huang, B. S. et al. (2018) The effectiveness of a saline mouth rinse regimen and education programme on radiation-induced oral mucositis and quality of life in oral cavity cancer patients: A randomised controlled trial. *European Journal of Cancer Care*. [Online]

Huang, C. J. et al. (2019) Randomized double-blind, placebo-controlled trial evaluating oral glutamine on radiation-induced oral mucositis and dermatitis in head and neck cancer patients. *American Journal of Clinical Nutrition*. [Online]

Humphrey, S. P. & Williamson, R. T. (2001) A review of saliva Normal composition, flow, and function. Humphrey, Williamson. 2001. Journal of Prosthetic Dentistry.pdf. *The Journal of Prosthetic Dentistry*. [Online]

Hunter, L. (2013) Saliva and oral health, 4th edition. *British Dental Journal*. [Online]

Inoue, H. et al. (2008) Rheological properties of human saliva and salivary mucins. *Journal of Oral Biosciences*. [Online]

Irune, E. et al. (2014) Treatment-related dysgeusia in head and neck cancer patients. Cancer Treatment Reviews

Isemura, S. et al. (1984) Cystatin S: A cysteine proteinase inhibitor of human saliva. *Journal of Biochemistry*. [Online]

Izumi, M. et al. (2015) Secretion of salivary statherin is compromised in uncontrolled diabetic patients. *BBA Clinical*. [Online]

Jansma, J. et al. (1989) In vivo study on the prevention of postradiation caries. *Caries research*. [Online]

Janus, M. M. et al. (2017) Candida albicans alters the bacterial microbiome of early in vitro oral biofilms. *Journal of Oral Microbiology*. [Online]

Jawad, H. et al. (2015) A review of dental treatment of head and neck cancer patients, before, during and after radiotherapy: Part 1. British Dental Journal

Jensen, S. B. et al. (2010) A systematic review of salivary gland hypofunction and xerostomia induced by cancer therapies: Prevalence, severity and impact on quality of life. Supportive Care in Cancer

Jensen, S. B. et al. (2003) Xerostomia and hypofunction of the salivary glands in

cancer therapy. Supportive Care in Cancer

Jham, B. C. et al. (2009) A randomized phase III prospective trial of bethanechol to prevent mucositis, candidiasis, and taste loss in patients with head and neck cancer undergoing radiotherapy: a secondary analysis. *Journal of oral science*. [Online]

Jham, B. C. et al. (2008) Oral health status of 207 head and neck cancer patients before, during and after radiotherapy. *Clinical Oral Investigations*. [Online]

Jin, Shuai et al. (2018) Relationship between subjective taste alteration and weight loss in head and neck cancer patients treated with radiotherapy: A longitudinal study. *European Journal of Oncology Nursing*. [Online]

Joshi, V. K. (2010) Dental treatment planning and management for the mouth cancer patient. Oral Oncology

Jung, Y.-S. et al. (2019) Oral Health Status and Oral Health-related Quality of Life According to Presence or Absence of Mucositis in Head and Neck Cancer Patients. *Journal of Cancer Prevention*. [Online]

Kadhim, M. et al. (2013) Non-targeted effects of ionising radiation-Implications for low dose risk. Mutation Research - Reviews in Mutation Research

Kawamoto, T. et al. (2018) Comparison of xerostomia incidence after three-dimensional conformal radiation therapy and contralateral superficial lobe parotid-sparing intensity-modulated radiotherapy for oropharyngeal and hypopharyngeal cancer. *Auris Nasus Larynx*. [Online]

Kidd, E. A. M. & Fejerskov, O. (2004) ‘What constitutes dental caries? Histopathology of carious enamel and dentin related to the action of cariogenic biofilms’, in *Journal of Dental Research*. [Online]. 2004 p.

Kielbassa, A. M. et al. (1999) Correlation of transversal microradiography and microhardness on in situ-induced demineralization in irradiated and nonirradiated human dental enamel. *Archives of Oral Biology*. [Online]

Kielbassa, A. M. et al. (2000) Microradiographic and Microscopic Studies on in situ Induced Initial Caries in Irradiated and Nonirradiated Dental Enamel. *Caries Research*. [Online]

Kielbassa, A. M. et al. (2006) Radiation-related damage to dentition. Lancet Oncology

Kilian, M. et al. (2016) The oral microbiome - An update for oral healthcare professionals. *British Dental Journal*. [Online]

Kimoto, M. et al. (2006) A role of salivary carbonic anhydrase VI in dental plaque. *Archives of Oral Biology*. [Online]

Klein, J. et al. (2014) Health related quality of life in head and neck cancer treated with radiation therapy with or without chemotherapy: A systematic review. Oral Oncology

Kouloulias, V. et al. (2013) The treatment outcome and radiation-induced toxicity for patients with head and neck carcinoma in the IMRT era: A systematic review with dosimetric and clinical parameters. BioMed Research International

Kugler, J. et al. (1992) Secretion of salivary immunoglobulin a in relation to age, saliva flow, mood states, secretion of albumin, cortisol, and catecholamines in saliva. *Journal of Clinical Immunology*. [Online]

Kwong, D. L. W. et al. (2004) Intensity-modulated radiotherapy for early-stage nasopharyngeal carcinoma: A prospective study on disease control and preservation of salivary function. *Cancer*. [Online]

Laheij, A. M. G. A. et al. (2015) Proteins and peptides in parotid saliva of irradiated patients compared to that of healthy controls using SELDI-TOF-MS Oral Health. *BMC Research Notes*. [Online]

Larhed, A. W. et al. (1998) The influence of intestinal mucus components on the diffusion of drugs. *Pharmaceutical Research*. [Online]

Lee, A. et al. (2012) Bacterial and Salivary Biomarkers Predict the Gingival Inflammatory Profile. *Journal of Periodontology*. [Online]

Lee, J. Y. et al. (2007) Comparison of the composition of oral mucosal residual saliva with whole saliva. *Oral Diseases*. [Online]

Levine, M. J. et al. (1978) Specificity of salivary-bacterial interactions: role of terminal sialic acid residues in the interaction of salivary glycoproteins with

Streptococcus sanguis and Streptococcus mutans. *Infection and Immunity*.

Li, J. et al. (2004) Statherin is an in vivo pellicle constituent: Identification and immuno-quantification. *Archives of Oral Biology*. [Online]

Lieshout, H. F. J. & Bots, C. P. (2014) The effect of radiotherapy on dental hard tissue-a systematic review. Clinical Oral Investigations

Ligtenberg, A. J. M. et al. (2015) The effect of physical exercise on salivary secretion of MUC5B, amylase and lysozyme. *Archives of Oral Biology*. [Online]

Ligtenberg, A. J. M. & Almståhl, A. (2015) 'Xerostomia and the Oral Microflora', in *Dry Mouth*. [Online]. p.

Little, M. et al. (2012) Reducing xerostomia after chemo-IMRT for head-and-neck cancer: Beyond sparing the parotid glands. *International Journal of Radiation Oncology Biology Physics*. [Online]

Logan, R. M. et al. (2007) The role of pro-inflammatory cytokines in cancer treatment-induced alimentary tract mucositis: Pathobiology, animal models and cytotoxic drugs. Cancer Treatment Reviews

Lu, H. et al. (2019) Direct radiation-induced effects on dental hard tissue. *Radiation Oncology*. [Online]

Lynge Pedersen, A. M. & Belstrøm, D. (2019) The role of natural salivary defences in maintaining a healthy oral microbiota. *Journal of Dentistry*. [Online]

Magalhães, A. C. et al. (2011) Fluoride in dental erosion. *Monographs in Oral Science*. [Online]

Magister, Š. & Kos, J. (2013) Cystatins in immune system. Journal of Cancer

Makkonen, T. A. et al. (1987) Dental extractions in relation to radiation therapy of 224 patients. *International Journal of Oral and Maxillofacial Surgery*. [Online]

Mandl, T. & Ekberg, O. (2019) 'Dysphagia in systemic disease', in *Medical Radiology*. [Online]. p.

Maria, O. M. et al. (2017) Radiation-Induced Oral Mucositis. Frontiers in Oncology

Martinez, K. D. O. et al. (2007) Secretory A immunoglobulin, total proteins and

salivary flow in Recurrent Aphthous Ulceration. *Brazilian Journal of Otorhinolaryngology*. [Online]

Martini, D. et al. (2017) Cystatin S-a candidate biomarker for severity of submandibular gland involvement in Sjögren's syndrome. *Rheumatology (United Kingdom)*. [Online]

Martini, S. et al. (2019) Prospective assessment of taste impairment and nausea during radiotherapy for head and neck cancer. *Medical Oncology*. [Online]

McCullough, R. W. (2017) US oncology-wide incidence, duration, costs and deaths from chemoradiation mucositis and antimucositis therapy benefits. *Future Oncology*. [Online]

McGuire, Jacob D., Walker, M. P., Dusevich, V., et al. (2014) 'Enamel organic matrix: Potential structural role in enamel and relationship to residual basement membrane constituents at the dentin enamel junction', in *Connective Tissue Research*. [Online]. 2014 p.

McGuire, J. D., Mousa, A. A., et al. (2014) Extracts of irradiated mature human tooth crowns contain MMP-20 protein and activity. *Journal of Dentistry*. [Online]

McGuire, J. D., Gorski, J. P., et al. (2014) Type IV collagen is a novel DEJ biomarker that is reduced by radiotherapy. *Journal of Dental Research*. [Online]

McGuire, Jacob D., Walker, M. P., Mousa, A., et al. (2014) Type VII collagen is enriched in the enamel organic matrix associated with the dentin-enamel junction of mature human teeth. *Bone*. [Online]

McQuibban, G. A. et al. (2000) Inflammation dampened by gelatinase a cleavage of monocyte chemoattractant protein-3. *Science*. [Online]

Memtsa, P.-T. et al. (2017) Assessment of xerostomia and its impact on quality of life in head and neck cancer patients undergoing radiation therapy. *Molecular and Clinical Oncology*. [Online]

Mese, H. & Matsuo, R. (2007) Salivary secretion, taste and hyposalivation. Journal of Oral Rehabilitation

Messana, I. et al. (2008) Facts and artifacts in proteomics of body fluids. What

proteomics of saliva is telling us? Journal of Separation Science

Metz, C. E. (1978) Basic principles of ROC analysis. *Seminars in Nuclear Medicine*. [Online]

Meurman, J. H. et al. (2002) Salivary albumin and other constituents and their relation to oral and general health in the elderly. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics*. [Online]

Meurman, J. H. & Grönroos, L. (2010) Oral and dental health care of oral cancer patients: hyposalivation, caries and infections. Oral Oncology

Miller, C. S. et al. (2006) Salivary biomarkers of existing periodontal disease: A cross-sectional study. *Journal of the American Dental Association*. [Online]

Möller, P. et al. (2004) A prospective study of salivary gland function in patients undergoing radiotherapy for squamous cell carcinoma of the oropharynx. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics*. [Online]

Mothersill, C. et al. (2019) Relevance of non-targeted effects for radiotherapy and diagnostic radiology; A historical and conceptual analysis of key players. *Cancers*. [Online]

Münstedt, K. et al. (2019) Honey in the management of side effects of radiotherapy- or radio/chemotherapy-induced oral mucositis. A systematic review. Complementary Therapies in Clinical Practice

Murphy, B. A. & Deng, J. (2015) Advances in supportive care for late effects of head and neck cancer. Journal of Clinical Oncology

Mutahar, M. et al. (2017) The presence of acquired enamel pellicle changes acid-induced erosion from dissolution to a softening process. *Scientific Reports*. [Online]

Nagler, R. M. (2007) Altered salivary profile in heavy smokers and its possible connection to oral cancer. *International Journal of Biological Markers*. [Online]

Närhi, T. O. et al. (1994) Antimicrobial factors, sialic acid, and protein concentration in whole saliva of the elderly. *European Journal of Oral Sciences*. [Online]

NAVAZESH, M. (1993) Methods for Collecting Saliva. *Annals of the New York Academy of Sciences*. [Online]

Nguyen, C. T. et al. (2018) Salivary flow rate after conventional and intensity-modulated radiation therapy. *Journal of the American Dental Association*. [Online]

Niemi, L. D. & Johansson, I. (2004) Salivary Statherin Peptide-Binding Epitopes of Commensal and Potentially Infectious Actinomyces spp. Delineated by a Hybrid Peptide Construct. *Infection and Immunity*. [Online]

Nikitkova, A. E. et al. (2013) Taking the starch out of oral biofilm formation: Molecular basis and functional significance of salivary α-amylase binding to oral streptococci. Applied and Environmental Microbiology

Nomura, M. et al. (2013) Irsogladine maleate reduces the incidence of fluorouracil-based chemotherapy-induced oral mucositis. *Annals of Oncology*. [Online]

Nomura, Y. et al. (2012) Salivary biomarkers for predicting the progression of chronic periodontitis. *Archives of Oral Biology*. [Online]

Normando, A. G. C. et al. (2017) Biomarkers in the assessment of oral mucositis in head and neck cancer patients: a systematic review and meta-analysis. Supportive Care in Cancer

Nutting, C. M. et al. (2011) Parotid-sparing intensity modulated versus conventional radiotherapy in head and neck cancer (PARSPORT): A phase 3 multicentre randomised controlled trial. *The Lancet Oncology*. [Online]

Oates, J. E. et al. (2007) Prospective evaluation of quality of life and nutrition before and after treatment for nasopharyngeal carcinoma. *Archives of Otolaryngology - Head and Neck Surgery*. [Online]

Obuchowski, N. A. (2003) Receiver operating characteristic curves and their use in radiology. Radiology

Ogama, N. et al. (2010) Appetite and adverse effects associated with radiation therapy in patients with head and neck cancer. *European Journal of Oncology Nursing*. [Online]

De Oliveira Mota, C. C. B. et al. (2013) Optical Coherence Tomography as an Auxiliary Tool for the Screening of Radiation-Related Caries. *Photomedicine and Laser Surgery*. [Online]

Orlandi, E. et al. (2018) Multivariable model for predicting acute oral mucositis during combined IMRT and chemotherapy for locally advanced nasopharyngeal cancer patients. *Oral Oncology*. [Online]

Osailan, S. M. et al. (2012) Clinical assessment of oral dryness: Development of a scoring system related to salivary flow and mucosal wetness. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology*. [Online]

Osman, T. A. et al. (2012) The use of salivary cytokines as a screening tool for oral squamous cell carcinoma: A review of the literature. Journal of Oral and Maxillofacial Pathology

Öztürk, L. K. et al. (2008) Association between dental-oral health in young adults and salivary glutathione, lipid peroxidation and sialic acid levels and carbonic anhydrase activity. *Brazilian Journal of Medical and Biological Research*. [Online]

Pacholke, H. D. et al. (2005) Late xerostomia after intensity-modulated radiation therapy versus conventional radiotherapy. American Journal of Clinical Oncology: Cancer Clinical Trials

Park, S. H. et al. (2004) Receiver operating characteristic (ROC) curve: Practical review for radiologists. Korean Journal of Radiology

de Paula, F. et al. (2017) Overview of Human Salivary Glands: Highlights of Morphology and Developing Processes. Anatomical Record

Pedersen, A. M. L. et al. (2018) Salivary secretion in health and disease. Journal of Oral Rehabilitation

Peluso, G. et al. (2007) Proteomic study of salivary peptides and proteins in patients with Sjögren's syndrome before and after pilocarpine treatment. *Arthritis and Rheumatism*. [Online]

Peros, K. et al. (2011) Salivary microbial and nonmicrobial parameters in children with fixed orthodontic appliances. *Angle Orthodontist*. [Online]

Picco, D. D. C. R. et al. (2017) Children with a higher activity of carbonic anhydrase VI in saliva are more likely to develop dental caries. *Caries Research*. [Online]

Pinna, R. et al. (2015) Xerostomia induced by radiotherapy: An overview of the physiopathology, clinical evidence, and management of the oral damage. Therapeutics and Clinical Risk Management

Pitts, N. B. (2001) Clinical diagnosis of dental caries: A European perspective. Journal of Dental Education

Pitts, N. B. et al. (2017) Dental caries. *Nature Reviews Disease Primers*. [Online]

Pitts, N. B. & Stamm, J. W. (2004) 'International Consensus Workshop on Caries Clinical Trials (ICW-CCT) - Final consensus statements: Agreeing where the evidence leads', in *Journal of Dental Research*. [Online]. 2004 p.

Ployon, S. et al. (2016) The membrane-associated MUC1 improves adhesion of salivary MUC5B on buccal cells. Application to development of an in vitro cellular model of oral epithelium. *Archives of Oral Biology*. [Online]

Podzimek, S. et al. (2016) Salivary Markers for Periodontal and General Diseases. *Disease Markers*. [Online]

Porter, S. R. et al. (2010) Xerostomia in head and neck malignancy. Oral Oncology

Pow, E. H. N. et al. (2006) Xerostomia and quality of life after intensity-modulated radiotherapy vs. conventional radiotherapy for early-stage nasopharyngeal carcinoma: Initial report on a randomized controlled clinical trial. *International Journal of Radiation Oncology Biology Physics*. [Online]

Pramanik, R. et al. (2010) Protein and mucin retention on oral mucosal surfaces in dry mouth patients. *European Journal of Oral Sciences*. [Online]

Pringle, S. et al. (2013) Concise review: Adult salivary gland stem cells and a potential therapy for xerostomia. Stem Cells

Proctor, G. B. et al. (2005) Salivary proteins interact with dietary constituents to modulate tooth staining. *Journal of Dental Research*. [Online]

Proctor, G. B. (2016) The physiology of salivary secretion. Periodontology 2000

Proctor, G. B. & Carpenter, G. H. (2007) Regulation of salivary gland function by

autonomic nerves. Autonomic Neuroscience: Basic and Clinical

Prodan, A. et al. (2015) Interindividual variation, correlations, and sex-related differences in the salivary biochemistry of young healthy adults. *European Journal of Oral Sciences*. [Online]

Qing, P. et al. (2015) Effect of gamma irradiation on the wear behaviour of human tooth enamel. *Scientific Reports*. [Online]

Quinn, B. et al. (2008) Guidelines for the assessment of oral mucositis in adult chemotherapy, radiotherapy and haematopoietic stem cell transplant patients. *European Journal of Cancer*. [Online]

Raber-Durlacher, J. E. et al. (2010) Oral mucositis. Oral Oncology

Raber-Durlacher, J. E. et al. (2013) Systematic review of cytokines and growth factors for the management of oral mucositis in cancer patients. Supportive Care in Cancer

Ranc, H. et al. (2006) Friction coefficient and wettability of oral mucosal tissue: Changes induced by a salivary layer. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. [Online]

Randall, K. et al. (2013) Analysis of factors influencing the development of xerostomia during intensity-modulated radiotherapy. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology*. [Online]

Reed, R. et al. (2015) Radiotherapy effect on nano-mechanical properties and chemical composition of enamel and dentine. *Archives of Oral Biology*. [Online]

Richards, T. M. (2014) Radiation-induced salivary gland hypofunction: Pathogenesis and prevention (doctoral dissertation). *King's College London*.

Richards, T. M. et al. (2017) The effect of parotid gland-sparing intensity-modulated radiotherapy on salivary composition, flow rate and xerostomia measures. *Oral Diseases*. [Online]

Rieger, J. M. et al. (2012) Functional outcomes related to the prevention of radiation-induced xerostomia: Oral pilocarpine versus submandibular salivary gland transfer. *Head and Neck*. [Online]

Riley, P. et al. (2017) Pharmacological interventions for preventing dry mouth and salivary gland dysfunction following radiotherapy. Cochrane Database of Systematic Reviews

Roesink, J. M. et al. (2001) Qantitative dose-volume response analysis of changes in parotid gland function after radiotheraphy in the head-and-neck region. *International Journal of Radiation Oncology Biology Physics*. [Online]

Roland, N. et al. (2016) Tumour assessment and staging: United Kingdom National Multidisciplinary Guidelines. *The Journal of Laryngology & Otology*. [Online]

Rosenthal, D. I. & Trotti, A. (2009) Strategies for Managing Radiation-Induced Mucositis in Head and Neck Cancer. Seminars in Radiation Oncology

Ruo Redda, M. G. & Allis, S. (2006) Radiotherapy-induced taste impairment. Cancer Treatment Reviews

De Sanctis, V. et al. (2016) Mucositis in head and neck cancer patients treated with radiotherapy and systemic therapies: Literature review and consensus statements. Critical Reviews in Oncology/Hematology

Sandow, P. L. et al. (2006) Taste loss and recovery following radiation therapy. *Journal of Dental Research*. [Online]

Santos-Silva, A. R. et al. (2015) cGVHD-related caries and its shared features with other ‘dry-mouth’-related caries. *Brazilian Dental Journal*. [Online]

Schenkels, L. C. P. M. et al. (1995) Biochemical composition of human saliva in relation to other mucosal fluids. Critical Reviews in Oral Biology and Medicine

Schipper, R. G. et al. (2007) Saliva as research material: Biochemical, physicochemical and practical aspects. Archives of Oral Biology

Schuurhuis, J. M. et al. (2011) Efficacy of routine pre-radiation dental screening and dental follow-up in head and neck oncology patients on intermediate and late radiation effects. A retrospective evaluation. *Radiotherapy and Oncology*. [Online]

Schuurhuis, J. M. et al. (2016) Head and neck intensity modulated radiation therapy leads to an increase of opportunistic oral pathogens. *Oral Oncology*. [Online]

Sciubba, J. J. & Goldenberg, D. (2006) Oral complications of radiotherapy. Lancet Oncology

Scorsetti, M. et al. (2010) Early clinical experience with volumetric modulated arc therapy in head and neck cancer patients. *Radiation Oncology*. [Online]

Scrimger, R. A. et al. (2018) Combination of submandibular salivary gland transfer and intensity-modulated radiotherapy to reduce dryness of mouth (xerostomia) in patients with head and neck cancer. *Head and Neck*. [Online]

Sennhenn-Kirchner, S. et al. (2009) Dental therapy before and after radiotherapy - An evaluation on patients with head and neck malignancies. *Clinical Oral Investigations*. [Online]

SHAO, Z.-Y. et al. (2011) Effects of Intensity-modulated Radiotherapy on Human Oral Microflora. *Journal of Radiation Research*. [Online]

Silva, A. R. S. et al. (2009) Patterns of demineralization and dentin reactions in radiation-related caries. *Caries Research*. [Online]

Sim, C. P. C. et al. (2018) Xerostomia, salivary characteristics and gland volumes following intensity-modulated radiotherapy for nasopharyngeal carcinoma: a two-year follow up. *Australian Dental Journal*. [Online]

Sio, T. T. et al. (2016) 'Intensity modulated proton therapy versus intensity modulated photon radiation therapy for oropharyngeal cancer: First comparative results of patient-reported outcomes', in *International Journal of Radiation Oncology Biology Physics*. [Online]. 2016 p.

De Siqueira Mellara, T. et al. (2014) The effect of radiation therapy on the mechanical and morphological properties of the enamel and dentin of deciduous teeth-an in vitro study. *Radiation Oncology*. [Online]

Siqueira, W. L. et al. (2008) Proteome of human minor salivary gland secretion. *Journal of Dental Research*. [Online]

Soares, C. J., Roscoe, M. G., et al. (2011) Effect of gamma irradiation and restorative material on the biomechanical behaviour of root filled premolars. *International Endodontic Journal*. [Online]

Soares, C. J. et al. (2010) Effect of gamma irradiation on ultimate tensile strength of enamel and dentin. *Journal of Dental Research*. [Online]

Soares, C. J., Neiva, N. A., et al. (2011) Effects of chlorhexidine and fluoride on irradiated enamel and dentin. *Journal of Dental Research*. [Online]

Sonesson, M. et al. (2003) Minor salivary gland secretion in children and adults. *Archives of Oral Biology*. [Online]

Sonis, S. T. (2004a) A biological approach to mucositis. Journal of Supportive Oncology

Sonis, S. T. (2009) Mucositis: The impact, biology and therapeutic opportunities of oral mucositis. Oral Oncology

Sonis, S. T. (1998) Mucositis as a biological process: A new hypothesis for the development of chemotherapy-induced stomatotoxicity. *Oral Oncology*. [Online]

Sonis, S. T. (2011) Oral mucositis. Anti-Cancer Drugs

Sonis, S. T. et al. (2001) Oral mucositis and the clinical and economic outcomes of hematopoietic stem-cell transplantation. *Journal of Clinical Oncology*. [Online]

Sonis, S. T. (2013) Oral Mucositis in Head and Neck Cancer: Risk, Biology, and Management. *American Society of Clinical Oncology Educational Book*. [Online]

Sonis, S. T. (2004b) The pathobiology of mucositis. Nature Reviews Cancer

Spielmann, N. & Wong, D. (2011) Saliva: Diagnostics and therapeutic perspectives. Oral Diseases

Spotten, L. et al. (2016) Subjective taste and smell changes in treatment-naive people with solid tumours. *Supportive Care in Cancer*. [Online]

Springer, I. N. et al. (2005) Radiation caries-radiogenic destruction of dental collagen. *Oral Oncology*. [Online]

Sroussi, H. Y. et al. (2017) Common oral complications of head and neck cancer radiation therapy: mucositis, infections, saliva change, fibrosis, sensory dysfunctions, dental caries, periodontal disease, and osteoradionecrosis. Cancer Medicine

Stoller, P. et al. (2002) Polarization-modulated second harmonic generation in collagen. *Biophysical Journal*. [Online]

Strojan, P. et al. (2017) Treatment of late sequelae after radiotherapy for head and neck cancer. Cancer Treatment Reviews

Sultani, M. et al. (2012) Anti-Inflammatory Cytokines: Important Immunoregulatory Factors Contributing to Chemotherapy-Induced Gastrointestinal Mucositis. *Chemotherapy Research and Practice*. [Online]

Sun, H. et al. (2019) Effects of a compound vitamin B mixture in combination with GeneTime® on radiation-induced oral mucositis. *Journal of International Medical Research*. [Online]

Takahashi, N. & Nyvad, B. (2008) Caries ecology revisited: Microbial dynamics and the caries process. Caries Research

Takahashi, N. & Nyvad, B. (2011) The role of bacteria in the caries process: Ecological perspectives. Journal of Dental Research

Takehara, S. et al. (2013) Degradation of MUC7 and MUC5B in Human Saliva. *PLoS ONE*. [Online]

Tharwat, A. (2018) Classification assessment methods. *Applied Computing and Informatics*. [Online]

Tschoppe, P. et al. (2010) Etiologic factors of hyposalivation and consequences for oral health. *Quintessence international (Berlin, Germany : 1985)*.

Turner, L. et al. (2013) Review of the complications associated with treatment of oropharyngeal cancer: a guide for the dental practitioner. *Quintessence international (Berlin, Germany : 1985)*. [Online]

Ukkonen, H. et al. (2017) Oral mucosal epithelial cells express the membrane anchored mucin MUC1. *Archives of Oral Biology*. [Online]

Valdez, I. H. et al. (1993) Major salivary gland function in patients with radiation-induced xerostomia: Flow rates and sialochemistry. *International Journal of Radiation Oncology, Biology, Physics*. [Online]

Vanhoecke, B. et al. (2015) Microbiota and their role in the pathogenesis of oral

mucositis. Oral Diseases

Vasconcelos, R. M. et al. (2016) Host-Microbiome Cross-talk in Oral Mucositis. Journal of Dental Research

Veerman, E. C. et al. (1989) Viscosity of human salivary mucins: effect of pH and ionic strength and role of sialic acid. *Journal de biologie buccale*.

Veness, M. J. et al. (2006) Use of topical misoprostol to reduce radiation-induced mucositis: Results of a randomized, double-blind, placebo-controlled trial. *Australasian Radiology*. [Online]

Vera-Llonch, M. et al. (2007) Oral mucositis and outcomes of allogeneic hematopoietic stem-cell transplantation in patients with hematologic malignancies. *Supportive Care in Cancer*. [Online]

Vera-Llonch, M. et al. (2006) Oral mucositis in patients undergoing radiation treatment for head and neck carcinoma. *Cancer*. [Online]

Vergeer, M. R. et al. (2009) Intensity-Modulated Radiotherapy Reduces Radiation-Induced Morbidity and Improves Health-Related Quality of Life: Results of a Nonrandomized Prospective Study Using a Standardized Follow-Up Program. *International Journal of Radiation Oncology Biology Physics*. [Online]

Vidotto, A. et al. (2010) Salivary and serum proteomics in head and neck carcinomas: Before and after surgery and radiotherapy. *Cancer Biomarkers*. [Online]

Vijay, A. et al. (2015) Factors that influence the extensional rheological property of saliva. *PLoS ONE*. [Online] 10 (8), .

Villa, A. et al. (2014) Diagnosis and management of xerostomia and hyposalivation. Therapeutics and Clinical Risk Management

Villa, A. & Sonis, S. T. (2015) Mucositis: Pathobiology and management. Current Opinion in Oncology

Villa, A. & Sonis, S. T. (2016) Pharmacotherapy for the management of cancer regimen-related oral mucositis. Expert Opinion on Pharmacotherapy

Vissink, A. et al. (2010) Clinical management of salivary gland hypofunction and

xerostomia in head-and-neck cancer patients: Successes and barriers. International Journal of Radiation Oncology Biology Physics

Vissink, A. et al. (2015) Current ideas to reduce or salvage radiation damage to salivary glands. *Oral Diseases*. [Online]

Vissink, A. et al. (2003) Oral sequelae of head and neck radiotherapy. Critical Reviews in Oral Biology and Medicine

Vitorino, R. et al. (2005) The role of salivary peptides in dental caries. *Biomedical Chromatography*. [Online]

Vukosavljevic, D. et al. (2014) Acquired pellicle as a modulator for dental erosion. Archives of Oral Biology

Walker, M. P. et al. (2011) Impact of radiotherapy dose on dentition breakdown in head and neck cancer patients. *Practical Radiation Oncology*. [Online]

Wang, X. & Eisbruch, A. (2016) 'IMRT for head and neck cancer: Reducing xerostomia and dysphagia', in *Journal of Radiation Research*. [Online]. 2016 p.

Wang, Z. et al. (2016) Minor salivary glands function is decreased in hyposalivation-related diseases. *Archives of Oral Biology*. [Online]

Warner, T. F. C. S. et al. (1985) Immunocytochemistry of acinic cell carcinomas and mixed tumors of salivary glands. *Cancer*. [Online]

Wiener, R. C. et al. (2010) Hyposalivation and xerostomia in dentate older adults. *Journal of the American Dental Association*. [Online]

Wijers, O. B. et al. (2002) Patients with head and neck cancer cured by radiation therapy: A survey of the dry mouth syndrome in long-term survivors. *Head and Neck*. [Online]

Willing, B. P. et al. (2011) Shifting the balance: Antibiotic effects on host-microbiota mutualism. Nature Reviews Microbiology

Wissinger, E. et al. (2014) The Economic Burden of Head and Neck Cancer: A Systematic Literature Review. PharmacoEconomics

Wong, T. K. (2011) The search on an ideal disease marker for childhood obstructive sleep apnea syndrome. Sleep

Xu, D. et al. (2016) Recent evolution of the salivary mucin MUC7. *Scientific Reports*. [Online]

Yavuz, B. & Bal Yılmaz, H. (2015) Investigation of the Effects of Planned Mouth Care Education on the Degree of Oral Mucositis in Pediatric Oncology Patients. *Journal of Pediatric Oncology Nursing*. [Online]

Zabernigg, A. et al. (2010) Taste Alterations in Cancer Patients Receiving Chemotherapy: A Neglected Side Effect? *The Oncologist*. [Online]

Zhang, C. Z. et al. (2016) Saliva in the diagnosis of diseases. International Journal of Oral Science

Zhou, X. H. et al. (2011) *Statistical Methods in Diagnostic Medicine*. [Online].

Zussman, E. et al. (2007) Age- and flow-dependency of salivary viscoelasticity. *Journal of Dental Research*. [Online]

Zwier, N. et al. (2013) Saliva parameters and erosive wear in adolescents. *Caries Research*. [Online]

Appendixes

Appendix 1: Research Ethics Committee approval letter

North of Scotland Research Ethics Service
Summerfield House
2 Eday Road
Aberdeen
AB15 6RE

Telephone: 01224 558468
Facsimile: 01224 558609
Email: nosres@nhs.net

NHS Grampian

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

02 November 2016

Professor Avijit Banerjee
Professor of Cariology & Operative
Conservative Department
Guys Hospital
Floor 26, Tower Wing
LONDON
SE1 9RT

Dear Professor Banerjee

Study title:	**"The characterisation of therapeutic radiation-induced dental caries; the histopathological, ultrastructural and microbial changes affecting its prevention and treatment"**
REC reference:	**16/NS/0116**
IRAS project ID:	**199100**

Thank you for your letter of 01 November 2016, responding to the Proportionate Review Sub-Committee's request for changes to the documentation for the above study.

The revised documentation has been reviewed and approved by the sub-committee.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager Miss Karen Gauld, nosres@nhs.net. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for HRA Approval (England)/ NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" above).

Approved documents

The documents reviewed and approved by the Committee are:

Document	*Version*	*Date*
Contract/Study Agreement	23 05 2016	30 September 2016
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) Indemnity policy	version 2	25 October 2016
IRAS Application Form	199100/1018524/37/594	18 October 2016
IRAS Checklist XML		31 October 2016
IRAS Application Form XML file		18 October 2016
Research protocol or project proposal	version 1	18 October 2016
Letter from statistician	23 05 2016	29 September 2016
TWICMC letter	23 05 2016	15 July 2015
GP letter HNC cross sect.	version 1	18 October 2016
GP letter control	version 1	18 October 2016
General Dental Practitioner letter	version 2	25 October 2016
General Dental Practitioner - Healthy Controls	version 2	25 October 2016
GP/consultant information sheets or letters - GP letter HNC follow up	version 1	18 October 2016
GP/consultant information sheets or letters - Gen Dental Practitioner letter	version 2	25 October 2016
Participant Information Sheet - HNC once	version 1	18 October 2016
Participant information sheet - H&N cancer	version 2	25 October 2016
Participant Information Sheet - H&N cancer cross sect	version 2	25 October 2016
Participant Information Sheet - Healthy Control	version 2	25 October 2016
Response to Provisional Opinion	version 1	31 October 2016
Participant consent form	version 2	25 October 2016
Referee's report or other scientific critique report		06 October 2016
Review	version 1	06 October 2016
Sample diary card/patient card - sample card	version 1	18 October 2016
Summary CV for Chief Investigator (CI)	version 1	18 October 2016
Summary CV for student	version 1	18 October 2016
Summary CV for supervisor (student research)	version 1	18 October 2016
Summary, synopsis or diagram (flowchart) of protocol in non technical language - flow chart	version 1	18 October 2016

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

16/NS/0116	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

Professor Nigel Webster
Chair

Enclosures: *"After ethical review – guidance for researchers" [SL-AR2]*

Copy to: *director of research management keith Brennan*
Jennifer Boston, Guy's & St Thomas' Foundation NHS Trust

Appendix 2: Research and Development approval letter

NHS
Health Research Authority

Miss Maria Gonzalez
229 Great Portland Stree
Guy's Hospital, Great Maze Pond
W1W 5PN

Email: hra.approval@nhs.net

21 January 2017

Dear Miss Gonzalez

Letter of HRA Approval

Study title:	**"The characterisation of therapeutic radiation-induced dental caries; the histopathological, ultrastructural and microbial changes affecting its prevention and treatment"**
IRAS project ID:	**199100**
REC reference:	**16/NS/0116**
Sponsor	**Kings College London and Guy's & Thomas' Foundation NHS Trust**

I am pleased to confirm that HRA Approval has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications noted in this letter.

Participation of NHS Organisations in England

The sponsor should now provide a copy of this letter to all participating NHS organisations in England.

Appendix B provides important information for sponsors and participating NHS organisations in England for arranging and confirming capacity and capability. **Please read *Appendix B* carefully**, in particular the following sections:

- *Participating NHS organisations in England* – this clarifies the types of participating organisations in the study and whether or not all organisations will be undertaking the same activities
- *Confirmation of capacity and capability* - this confirms whether or not each type of participating NHS organisation in England is expected to give formal confirmation of capacity and capability. Where formal confirmation is not expected, the section also provides details on the time limit given to participating organisations to opt out of the study, or request additional time, before their participation is assumed.
- *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* - this provides detail on the form of agreement to be used in the study to confirm capacity and capability, where applicable.

Further information on funding, HR processes, and compliance with HRA criteria and standards is also provided.

It is critical that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details and further information about working with the research management function for each organisation can be accessed from www.hra.nhs.uk/hra-approval.

Appendices

The HRA Approval letter contains the following appendices:

- A – List of documents reviewed during HRA assessment
- B – Summary of HRA assessment

After HRA Approval

The document *"After Ethical Review – guidance for sponsors and investigators"*, issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:

- Registration of research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

In addition to the guidance in the above, please note the following:

- HRA Approval applies for the duration of your REC favourable opinion, unless otherwise notified in writing by the HRA.
- Substantial amendments should be submitted directly to the Research Ethics Committee, as detailed in the *After Ethical Review* document. Non-substantial amendments should be submitted for review by the HRA using the form provided on the HRA website, and emailed to hra.amendments@nhs.net.
- The HRA will categorise amendments (substantial and non-substantial) and issue confirmation of continued HRA Approval. Further details can be found on the HRA website.

Scope

HRA Approval provides an approval for research involving patients or staff in NHS organisations in England.

If your study involves NHS organisations in other countries in the UK, please contact the relevant national coordinating functions for support and advice. Further information can be found at http://www.hra.nhs.uk/resources/applying-for-reviews/nhs-hsc-rd-review/.

If there are participating non-NHS organisations, local agreement should be obtained in accordance with the procedures of the local participating non-NHS organisation.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application

IRAS project ID	199100

procedure. If you wish to make your views known please email the HRA at hra.approval@nhs.net. Additionally, one of our staff would be happy to call and discuss your experience of HRA Approval.

HRA Training

We are pleased to welcome researchers and research management staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

Your IRAS project ID is **199100**. Please quote this on all correspondence.

Yours sincerely

Beverley Mashegede
Assessor

Email: hra.approval@nhs.net

Copy to: Keith Brennan (Kings College London), Sponsor Contact
Mrs Jennifer Boston (Guy's & St Thomas' Foundation Trust), Lead NHS R&D Contact

Guy's and St Thomas' NHS
NHS Foundation Trust

Research & Development
16th Floor Tower Wing
Guy's Hospital
Great Maze Pond
London SE1 9RT
Tel: 020 7188 7188

Conditions of confirmation of capacity and capability to conduct research at Guy's and St Thomas' NHS Foundation Trust (GSTFT)

1. **Recruitment reporting:**
   It is mandatory that all research participants at Guy's and St Thomas' NHS FT are entered into the research management system 'Edge'. Your R&D contact will have created you (or your nominated delegate) an account for the system. Please refer to the 'Quick Help Guide' already provided. Participants must be uploaded into the system in real-time or weekly as a minimum, in order for the Trust to maintain a real-time record of its recruitment into clinical research studies. Please contact edgesupport@gstt.nhs.uk if you have any problems. The R&D department hosts regular training sessions on the system. Please enquire about these via the same e-mail.

2. **Safety reporting**
   All investigators should ensure that they elicit information regarding adverse events from participants at each study visit. If a Serious Adverse Event (SAE) is discovered, the investigator must alert the Sponsor immediately (within 24 hours) to ensure that events are reported to ethics and regulatory bodies within the timelines.

   Please refer to the HRA guidance on safety reporting:
   http://www.hra.nhs.uk/research-community/during-your-research-project/safety-reporting/

   For Trust/KCL sponsored Clinical Trials of Investigational Medicinal Products, please refer to the King's Health Partner Clinical Trials Office Standard Operating Procedures:
   http://www.khpcto.co.uk/SOP/SOP.html

3. **Protocol deviations and breaches in GCP**
   If there is a breach of the study protocol or Good Clinical Practice, likely to affect to a significant degree:
   - the safety or physical or mental integrity of the subjects of the trial and or;
   - the scientific value of the trial

   This must be reported within 24 hours to the R&D department.

   For Trust/KCL sponsored Clinical Trials of Investigational Medicinal Products, please refer to the King's Health Partner Clinical Trials Office Standard Operating Procedures:
   http://www.khpcto.co.uk/SOP/SOP.html

4. **Protocol Amendments**
   Investigators should alert the R&D Department if there is an amendment to the study. An amendment may include changes to study documentation, a decision to use advertising, changes to staff or revisions to study timelines.

   Where studies are sponsored by GSTFT, changes to the protocol and/or other study documents must be submitted to the R&D Department for review prior to ethics submission if applicable. Trust confirmation of capacity and capability must be issued prior to the implementation of any amendment.

5. **Training**
   Please ensure all researchers for the study have the appropriate study specific training. For Clinical Trials of Investigational Medicinal Products, each member of the team is required to have up to date training on Good

Appendix 3: Clinical trial participant's information sheet

Information sheet

1. Study title

"The characterisation of therapeutic radiation-induced dental caries: the histopathological, ultra-structural and microbial changes affecting its prevention and treatment"

2. Invitation paragraph

You are being invited to take part in a research study. Before you decide whether or not to participate, it is important for you to understand why the research is being carried out and what it will involve. Please take the time to read the following information carefully and discuss it with friends, relatives and your general dental practitioner. Please ask us if there is anything that is not clear or if you would like more information.

Take the time to decide whether or not you wish to take part. Thank you for reading this.

3. What is the purpose of the study?

The first treatment option in head and neck cancer is radiation therapy, which entails, in simple words, high-energy X-rays. All of the head and neck cancer patients who take part in this study are going to receive this therapy, with the dose and duration of the treatment being determined by the oncologist; the exposure that you will receive as part of your care will not exceed the normal dose. As part of your treatment pathway, you are going to attend a dental appointment at Guy's Hospital (on floor 26) and receive routine dental X-rays, which involve small exposure to ionising radiation; this is not above what would be received routinely as part of your dental treatment before starting cancer therapy.

Patients treated for head and neck cancer may suffer both short-term and long-term side effects around the area for which they are receiving the therapy, which can include speech defects, difficulties in eating, dry mouth, dental problems, sore mouth, and limitation of mouth opening.

The aim of the study is to identify oral side effects of radiotherapy for head and neck cancer patients in order to assess whether specific changes in oral microbiota, saliva quality and teeth following radiation therapy are associated with both the severity and the increased risk of caries.

We will also follow up with participants at 12 months post-enrolment in the trial. At their routine follow-up appointments we will add two more visits after they finish their regular dental appointments at the special dental care clinic at Guy's Hospital.

If successful, outcomes of this project will be used to change the service that we offer to patients to improve patients' experience of treatment and to improve patients' long-term quality of life.

This study is being carried out in partial fulfilment of the requirements for the degree of Doctor of Philosophy (PhD) of Ms. Maria Ines Gonzalez Agurto.

4. Why have I been chosen?

We are inviting you to take part in this study because you are about to start treatment for head and neck cancer. This makes you a suitable person for this study.

5. Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care that you receive in any way.

6. What will happen to me if I take part?

If you decide to take part in this research, your treatment for cancer and your dental treatment will proceed as normal. There will be a further two visits, and the two regular visits to the special dental care clinic will be slightly longer.

The aim of the study is to find out the most appropriate treatment regime to limit side effects of radiotherapy on oral health, therefore improving patients' quality of life after treatment for head and neck cancer.

We will follow up with participants at 18 months post-enrolment in the trial. At your appointments, we will conduct a clinical evaluation of the dental status and periodontal status. After an initial oral evaluation, samples of whole mouth saliva will be collected. Supragingival dental plaque, a mucosal swab from the inner cheek and, finally, carious dentine samples will be collected using two different methods: either from extracted teeth or directly from dentinal carious lesions of teeth that will be restored.

This will be repeated three times, after the completion of cancer treatment. This would involve four extra visits for patients beyond current practice.

7. What do I have to do?

Patients who agree to take part in this study will be required to attend four extra visits to Guy's Hospital for follow-up during an 18-month period, which are free of charge.

8. What is the drug or procedure that is being tested?

We are not testing any new procedure or material.

9. What are the side effects of taking part?

There are no side effects of taking part other than those expected from routine dental care.

10. What are the possible disadvantages and risks of taking part?

There are no risks in taking part. The only disadvantage of taking part is a slightly longer-than-normal dental appointment of perhaps an extra 30 minutes approximately.

11. What are the possible benefits of taking part?

There are no advantages of taking part. The information that we obtain from this study may help us to treat future patients better.

12. What if something goes wrong?

If you have a concern about any aspect of this study, you should ask to speak to the researchers, who will do their best to answer your questions. Please contact the principal investigator: Professor Avijit Banerjee, avijit.banerjee@kcl.ac.uk, telephone number (+44) 07470223082. If you remain unhappy and wish to complain formally, you can do this through the Guy's and St Thomas' Patients Advice and Liaison Service (PALS) on 020 7188 8801, pals@gstt.nhs.uk. The PALS team are based in the main entrance on the ground floor at St Thomas' Hospital and on the ground floor at Guy's Hospital in the Tower Wing.

In the event that something does go wrong and you are harmed during the research, you may have grounds for legal action for compensation against Guy's and St Thomas' NHS Foundation Trust and/or King's College London, but you may have to pay for legal costs. The normal National Health Service complaint mechanisms will still be available to you (if appropriate).

13. Will my taking part in this study be kept confidential?

All information collected during the course of the research will be kept strictly confidential. We would also inform your general dental practitioner about your health with your permission.

14. What will happen to the results of the research study?

Results of this research will be published in appropriate dental and scientific journals. No personal information or other information that could be identified as relating to you will be published. You will be informed of the results of the study.

15. What will happen to my samples?

All of the samples collected from you would be labelled and stored in the microbiology laboratory on the Waterloo campus for further microbiological and biochemical analysis. The teeth collected will be placed in the bio-photonic laboratory for further microscopy image analysis. The samples will be disposed according to the Human Tissue Act protocol.

16. Where my personal data will be kept?

The Principal Investigator will maintain a database of the patient's personal data clinical notes and treatment records. The data will not be transferred outside UK and will be analysed in the chief investigator room.

Physical personal data collected in the course of research will be stored under controlled access in a locked cabinet, in a clear filing systems in chief investigator office on 26 floor at Guy's campus. (Safe from flood, fire, burglary and pest)

17. How long will personal data be stored or accessed after the study has ended?
The data will be stored 3 years, after that time the data will be destroyed.

18. How long will research data generated by the study be stored?
The data generated in this study will be stored 5 years after start of the study.

19. Who is organising and funding the research?
King's College London.

20. Who has reviewed the study?
North of Scotland Research Ethics Committee London – South East reviewed this study.

21. Contacts for further information
For further information please contact:
Avijit Banerjee
PhD student: Maria Ines Gonzalez
King's College London Dental Institute
Biomaterials Research Group
Floor 17, Guy's Tower

Appendix 5: Clinical trial consent form

KING'S College LONDON

Guy's and St Thomas' NHS
NHS Foundation Trust

CONSENT FORM FOR PARTICIPANTS IN RESEARCH STUDIES.

Title of Project: The characterisation of therapeutic radiation-induced dental caries; the histopathological, ultra-structural and microbial changes affecting its prevention and treatment"

Name of Researcher: Avijit Banerjee

Thank you for considering taking a part in this research. The person organising the research must explain the project to you before you agree to take a part. If you have any question arising from the information sheet or explanation already given to you, please ask the researcher before you decide whether to join in. You will be given a copy of this consent form to keep and refer to any time.

Please Tick or Initial

I confirm that I understand that by ticking/initialling each box I am consenting to this element of the study. I understand that it will be assumed that unticked/initialled boxes mean that I DO NOT consent to that part of the study. I understand that by not giving consent for any one element I may be deemed ineligible for the study. ☐

1. I confirm that I have read and understand the information sheet dated 25 October 2016 Version 2 for the above study and I have had the opportunity to ask questions, which have been answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from [Guy's hospital] or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I consent to the processing of my personal information for the purposes explained to me. I understand that such information will be handled in accordance with the terms of the UK Data Protection Act 1998 ☐
5. I understand that confidentiality and anonymity will be maintained and it will not be possible to identify me in any publications ☐
6. I understand that the research team may use registered external organisations for the processing of my data and that in such cases my confidentiality and anonymity will be maintained. ☐

7. I agree that the research team may use my data for future research and understand that any such use of identifiable data would be reviewed and approved by a research ethics committee. (In such cases, as with this project, data would not be identifiable in any report). ☐
8. I agree that the research team contact my general dental practitioner to inform that I agreed to participate in this study. ☐

I agree that the research project named above has been explained to me to my satisfaction and I agree to take part in the study. I have read both the notes written above and the Information Sheet about the project, and understand what the research study involves. ☐

Name of Patient	Signature	Date
Researcher	Signature	Date

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Appendix 6: Clinical trial patient sample card

Patient sample card

Name

Age

Mobile

Email Gender F M

Type of Ca

Treatment Date of treatment

GDP information

Sample collection	AFTER RT / CONTROL Date
DMFS Type of teeth ICDAS White spot Dentin	
UWMSS	
Mouth rinse	
Biofilm	
Carious dentine/teeth number	
Mucosal swab	

Version 1 18 October 2016 IRAS 199100

Follow up

Sample collection	Visit 1 PRE RT DATE	Visit 2 (6months) DATE	Visit 3 (12 months) DATE
Medication DMFS Type of teeth ICDAS White spot Dentin			
UWMSS	Weight:		
Mouth rinse			
Biofilm	Weight: Number:		
Carious dentine/teeth Number	Weight: Number:		
Surface Pain	O M D V P/L Yes No:		
Mucosal swab	1 2		

Appendix 7: KDPR letter

Research Governance

Franklin Wilkins Building
Waterloo Bridge Wing
Waterloo Road
London SE1 9NH
Telephone 020 7848 3333
gdpr@kcl.ac.uk

KING'S College LONDON

01/05/2018

Maria Gonzalez Agurto

Dear Maria

KDPR Registration Reference: DPRF-17/18-8377
Project Title: Radiation Induced Dental caries

Thank you for submitting the above Research Data Protection Registration Form. This letter acknowledges confirmation of your registration; your registration confirmation reference number is detailed above.

Be sure to keep a record your registration number. A copy of this letter will automatically be stored in your KDPR account, but you may wish to keep a separate copy in your own records.

Registration is valid for the data holding period you have indicated within the form.

Please note it is the responsibility of the researcher to ensure that any other permissions or approvals (i.e. Research Ethics, R&D, gatekeepers, etc.) relevant to their research are in place, prior to data collection.

Modifications

Should there be any changes to the conduct of your study or your study timelines which will impact on how you collect, manage or otherwise use your data, then you must submit a modification request in KDPR, indicating what has changed. Modification requests will be required in instances such as (this is not an exhaustive list):

- Change of storage repository
- Change to data retention period
- Change of data controller if that person should leave the College
- Change to the nature of the identifiers in the data you collect

You will find the modification request form within the project you have created. You can access this by selected 'Create sub-form' in the left hand tiles on the screen and selecting 'Modification Request Form.'

Please note the modification request form will be available from Friday 27th April

Audit:

As part of the College's responsibilities under the General Data Protection Regulation, it must ensure that data is collected, managed and otherwise used as outlined within the submitted registration forms. As such the College is required to audit this process. You may therefore be selected for a random audit, to see how researchers are implementing this process. If audited, you will be expected to provide evidence that you are collecting, managing or otherwise using your data as outlined within the form.

If you have any questions regarding your registration please email gdpr@kcl.ac.uk

We wish you every success with your project.

With best wishes

KCL Research Governance Team

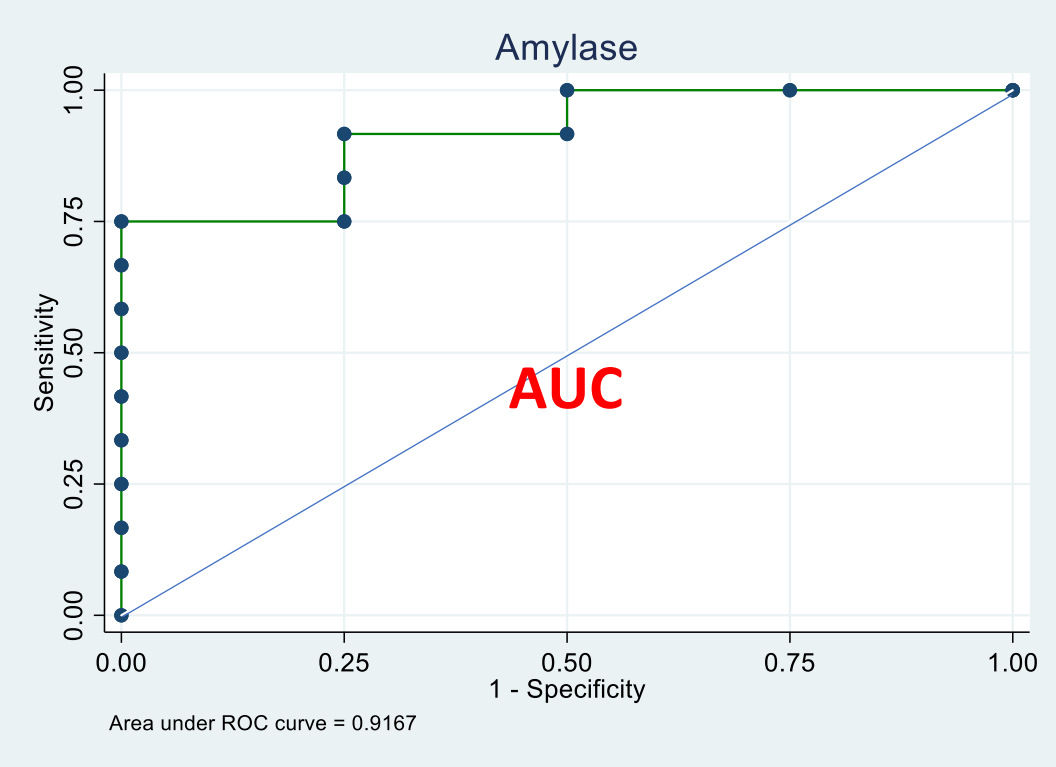
Page 1 of 1

Appendix 8: ROC curve Graph

ROC curve Graph

ROC curve graph is a visual tool for evaluating the diagnostic power of the test - in this case of selected salivary proteins (concentration\secretion rate) to predict oral mucositis onset and severity. ROC curve provides visual information regarding the relationship between sensitivity and specificity of every cut off point for each protein selected. Every cut off represents a different value for sensitivity and specificity, generating a point on the graph on the ROC curve that indicates whether a patient is likely to be diseased or not. The accuracy of the test is represented by the shape of the slope, which is more accurate when the curve is closer to the top and left-hand borders. The area under the curve (AU ROC) of a test can be used as a criterion to measure the test's discriminative power, the maximum value is 100%, on the other hand when the curve is closer to the diagonal located from the bottom left corner to the upper right of the graph is less accurate having a discriminatory power of 50 % (50 % sensitivity and specificity) which represents pure chance (Park et al., 2004; Zhou et al., 2011;Tharwat, 2018).

Analysis of amylase units/ ml collected at Time 0 as a predictor of Oral mucositis grade 1 and 2 two weeks post IMRT.



The graph of 2 weeks after IMRT shows that α-amylase unit concentration can be an accurate predictor of oral mucositis grades 1 and 2, with an area under the ROC curve of 91.67%, true-positive rate is high, and the false-positive rate is low, true Positive Rate represents the y-axis and False Positive Rate is the x-axis. α-amylase cut-off point that maximises sensitivity and specificity is > 22.77 units/ml, with a sensitivity of 100% and specificity of 50%.